Tetrahydroxy Flavone from Acacia auriculiformis A. Cunn Ex Benth. (Fabaceae) with Novel Kinase Activity

Augustine A. Ahmadu1*, Bilqis A. Lawal2, Anas Haruna1, Lukman Mustapha1

ABSTRACT
Background: The decoctions of the bark of Acacia auriculiformis are used in folkloric medicine to relieve pain and inflammation and as remedy for cancer. Objective: The aim of this work is to screen the extract and fractions of Acacia auriculiformis for protein kinase inhibitory activity and also to isolate and characterize chemical entities from this plant and evaluate their protein kinase inhibitory activity. Materials and Methods: Kinase inhibitory activity were assayed in appropriate buffer, with either protein or peptide as substrate in the presence of 15µM (33-P) ATP (3,000Ci/mmol; 10mCi/ml) in a final volume of 30µL. Controls were performed with appropriate dilutions of dimethyl sulfoxide. A portion of the Chloroform extract, ethylacetate and n-butanol soluble fractions of the stem bark of Acacia auriculiformis were screened against a panel of disease-related protein kinases and the active fractions was tested over a wide range of concentrations from 0.016 to 50µg/ml and the IC50 values were determined from the dose response curve. The most active fraction was subjected to chromatographic separation using silica gel G column chromatography and sephadex LH-20 to give compound I. The structure of the isolated compound was elucidated using NMR and LC-MS. Results: The primary screening of the extract and fractions showed that the chloroform extract was inactive against all the protein kinases investigated, while the ethylacetate and n-butanol soluble fractions inhibited all the protein kinases tested. Compound I also inhibited all the kinases tested. The IC50 of the active fractions and compound were also evaluated. Ethylacetate fraction inhibited all the kinases tested with the highest activity against Haspine kinase with IC50 of 1.0 µg/ml, while n-butanol also gave the highest activity against Haspine kinase with 1C50 of 1.3 µg/ml. From the active ethylacetate fractions 3, 4', 7, 8- tetrahydroxy flavone was isolated. The Compound exhibited the maximal activity against DYRK1A kinase with an IC50 of 2.06 µg/ml followed by CDK9 with an IC50 of 2.28 µg/ml. Conclusion: 3, 4',7, 8- tetrahydroxy flavone was isolated was found to be a DYRK1A and CDK9 inhibitor which might justify the anticancer potential of this plant. Key words: Tetrahydroxylavone, Protein kinases, CDK9, DYRK1A.

INTRODUCTION

Human protein kinases represent the third largest enzyme class and are responsible for modifying an estimated one-third of human proteome.1 It has been firmly demonstrated that hyperactivation, hyperproduction or mutations of these kinases leading to the disruption of cell signalling cascades, play important role in several diseases such as cancer, inflammation, diabetes among others, thus making protein kinases one of the most important targets for pharmaceutical industry.2 The human genome consists of over 517 enzyme class and are responsible for modifying an estimated one-third of human proteome.1 It has been firmly demonstrated that hyperactivation, hyperproduction or mutations of these kinases leading to the disruption of cell signalling cascades, play important role in several diseases such as cancer, inflammation, diabetes among others, thus making protein kinases one of the most important targets for pharmaceutical industry.2 The human genome consists of over 517 protein kinases that include two sub families, serine/threonine and tyrosine kinases.3 Numerous tyrosine kinases inhibitors have been discovered by screening plant extracts based on ethnopharmacological and chemotaxonomic knowledge.4 Specific screening approach have led to the isolation of structurally distinct classes of inhibitors which have served as leads for further design and synthesis of more active anologues.

Acacia is quite a large genus of the family fabaceae, with about 1,400 species. Most of the species belonging to the genus are rich in secondary metabolites containing mainly tannins, flavonoids and gums5 and is widely distributed in tropical and non tropical countries including Nigeria. Acacia auriculiformis also referred to as ear leaf Acacia is an important medicinal plant and widely distributed member of the fabaceae. An infusion of the bark of this plant is used to treat inflammation among the aborigines of Australia.6 The antimutagenic and chemoprotective activities of extracts of the stem bark of Acacia auriculiformis have been reported.7 The antioxidant and free radical scavenging properties of the ethylacetate and acetone fraction of the stem bark of this plant have been...
reported. A new triterpenoid trisaccharide and three new triterpenoids have been isolated from this plant. The antimicrobial activity of saponins and a new flavan glycoside have been reported from this plant. Previously, we have reported the protein kinase inhibitory activity of Acanthol 2, a new Peltogynoid isolated from the bark of Acacia nilotica and recently, we also reported that the ethylacetate soluble fraction and two compounds isolated from the fraction inhibited a panel of protein kinases and (+)-catechin showed tissue necrosis of some tumoral cell. In our continuing investigation of the genus acacia for protein kinase inhibitory constituents, we report herein the activity of the extract, fractions and the isolated flavonoid from the active ethylacetate soluble fraction of the stem bark of Acacia auriculiformis against a panel of some disease related protein kinases.

MATERIALS AND METHODS

General experimental procedures

Column chromatography was performed on silica gel G (200–400 mesh, Silicycle), Thin layer chromatography was performed on pre-coated TLC plate silica gel 0.2mm aluminium backed (Silicycle), NMR spectra was performed on a Bruker DRX 400 MHz spectrophotometer in CD3OD using TMS as internal standard, while LC-MS was carried out using an ESI-LTQ-orbitrap discovery XL mass spectrometry.

Plant material

The plant consisting of the leaves and stem bark were collected in Zaria in the month of July and were authenticated at the herbarium section of the Biological Science Department, Ahmadu Bello University, Zaria, where a voucher specimen was deposited.

Extraction and isolation

The dried pulverised stem bark (400 g) was extracted with chloroform (2X2L) at room temperature and the solvent removed at reduced pressure afforded a dark brown solid mass (3.5 g). A portion of the chloroform, ethylacetate and n-butanol fractions and the isolated flavonoid from the active ethylacetate soluble fraction of the stem bark of Acacia auriculiformis against a panel of some disease related protein kinases.

Protein kinase inhibitory studies

A portion of the chloroform, ethylacetate and n-butanol fractions and compound I were screened against a panel of diseased related protein kinases. Kinase activity were assayed in appropriate buffer, with either protein or peptide as substrate in the presence of 15µM (32P) ATP (3,000Ci/mmol; 10mCi/ml) in a final volume of 30µL following the assay as described. Controls were performed with appropriate dilutions of dimethyl sulfoxide. Full length Kinas were used unless specified. Peptide substrates were obtained from Proteogenix (Oberhausenbergen, France).

Buffers

A 10 mM MgCl2, 1mM EGTA, 1mM DTT, 25 mM Tris-HCl pH 7.5,50 µg/ml heparin.

B 60 mM β-glycerophosphate, 30mM p-nitrophenyl-phosphate, 25mM MOPS (pH7), 5mM EGTA, 15mM MgCl2, 1mM DTT, 0.1 mM sodium orthovanadate.

(D) 25 mM MOPS, pH7.2, 12.5 mM β-glycerophosphate, 25 mM MgCl2, 5mM EGTA, 2mM EDTA, 0.25 mM DTT.

(H) MOPS 25mM pH 7.5, 10mM MgCl2

(K) Tris 50mM pH 7.5, 20mM MgCl2, 2mM MnCl2.

(R) 5mM MOPS pH 7.2, 2.5 mM β-glycerophosphate, 4mM MgCl2, 2.5mM MnCl2, 1mM EGTA, 0.4mM EDTA,50 µg/ml BSA, 0.05 mM DTT.

Hs CDK2/Cyclin A (cyclin-dependent kinase-2, human, kindly provided by Dr. A. Echalier-Glazer, Leicester, UK) was assayed in buffer A (+0.15 mg/mL of BSA +0.23 mg/mL of DTT) with 0.8 µg/mL of histone H1 as substrate.

HsCDK9/Cyclin T (human, recombinant, expressed by baculo virus in S19 insect cells was assayed in buffer A (+0.15 mg/mL of BSA +0.23 mg/mL of DTT) with 0.27 µg/mL of the following peptide: YSPTPSYSPTPSKDKKKK, as substrate.

HsCDK5/p25 (human, recombinant, expressed in bacteria) was assayed in buffer B, with 0.8 µg/µL of histone H1 as substrate.

HsPIM1 (human proto-oncogene, recombinant, expressed in bacteria) was assayed in buffer B, 0.8 µg/µL of histone H1 (Sigma #H5505) as substrate.

HsHaspin-kd (human, kinase domain, amino acids 470 to 798, recombinant, expressed in bacteria) was assayed in buffer H with 0.007 µg/µL of Histone H3 (1-21) peptide (ARTKQTARKSTGGKAPRKQLA) as substrate.

HsRIPK3 (human, recombinant, expressed in Sf9 insect cells) was assayed in buffer R with 0.1 µg/µL of MBP as substrate.

HsAuroraB (human, recombinant, expressed by baculovirus in S19 insect cells, Signal Chem, product #A31-10G) was assayed in buffer D with 0.2 µg/µL of MBP as substrate.

ScsGSK3-α,β (glycogen synthase kinase-3, porcine brain, native, affinity purified) was assayed in buffer A (+0.15 mg/mL of BSA +0.23 mg/mL of DTT ), with 0.010 µg/mL of GS-1 peptide, a GSK-3 selective substrate.

ScsGSK3-α,β (glycogen synthase kinase-3, porcine brain, native, affinity purified) was assayed in buffer A (+0.15 mg/mL of BSA +0.23 mg/mL of DTT ) with 0.033 µg/mL of the following peptide: RRKHAAGIsPSYISTA as substrate.

KnDYRK1A-kd (Rattus norvegicus, amino acids 1 to 499 including the kinase domain, recombinant, expressed in bacteria, DNA vector provided by Dr W. Becker, Aachen, Germany) was assayed in buffer A (+0.5 mg/mL of BSA +0.23 mg/mL of DTT ) with 0.033 µg/mL of the following peptide: KKKISGRLSIPMTEQ as substrate.

MmClk1 (from Mus musculus, recombinant, expressed in bacteria) was assayed in buffer A (+0.15 mg/mL of BSA +0.23 mg/mL of DTT) with 0.027 µg/mL of the following peptide: RRKKHRASPSYISTA as substrate.

PgGSK-3 (from Plasmodium falciparum, recombinant, expressed in bacteria) was assayed in Buffer A (+0.15 mg/mL of BSA +0.23 mg/mL of DTT) with 0.010 µg/mL of GS-1 peptide, a GSK-3-selective substrate.
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(3.4VAPPSSPSHSSPQaSPEDEEE, sp stands for phosphorylated serine).

LdTLK (tousled-like kinase, from Leishmania donovani, recombinant, expressed in bacteria), was assayed in buffer K with 0.6 µg/µL of casein dephosphorylated from bovine milk (Sigma #C4032) as substrate.

LmCK1 (from Leishmania major, recombinant, expressed in bacteria) was assayed in buffer B (adjusted at pH 8) with 0.028 µg/µL of the following peptide: RKKHAAIGSpAYSITA as CK1-specific substrate.

RESULTS

Compound 1 was isolated as a yellow solid (3 mg). Its spectral data (1H-, NMR and LC-MS) are consistent with literature data for 3, 4', 7, 8'-tetrahydroxyflavone.

Effects of the extract/fractions on the activity of disease-related protein kinases

Table 1 shows the results of the primary screening of the chloroform extract, ethyl acetate-n-butanol soluble fractions and compound 1 against a panel of protein kinases. The result indicates the activity that remains in the tube after treating the mentioned kinase with 50µg/mL of the extract/fraction compared to control assay treated with DMSO. The result revealed that the chloroform extract showed the least activity against the kinases as the extract did not inhibit the activity of the kinases and hence the high % activity of each kinase remaining in the tube this can be observed on the activity of the extract against Pim1 which showed 91% of the activity of the kinase still remaining in the tube after treatment with 50µg/mL of the extract in comparison with the control. The ethyl acetate and n-butanol fractions in contrast, however showed a reduction in kinase activity in the tube after treating the mentioned extract with 50µg/mL of the fraction. In Table 1, the ethyl acetate fraction on Pim1 showed only 2% of remaining activity of the kinase in the tube compared to DMSO. A similar trend was observed in n-butanol fraction. The isolated flavonoid also shows some inhibitory activity against all the kinases tested. The ethyl acetate, n-butanol fractions and compound 1 were next evaluated over a wide range of concentrations from 0.016µg/mL to 50µg/mL and IC50 values were determined from the dose response curves (Sigma-plot). The results of the in vitro enzymatic activity assay were summarised in Table 2.

Table 2 shows the inhibitory activity of the ethyl acetate, n-butanol fractions and isolated flavonoid against the mentioned kinases with their IC50. The result revealed that the ethyl acetate fraction is the most active with an IC50 of 1.0µg/mL against Haspine kinase followed with Hs CDK9 with an IC50 of 4.5µg/mL. The activity of the ethylacetate fraction is more than n-butanol fraction.

DISCUSSION

Compound 1 was isolated as a yellow solid, its mass spectral data gave a molecular ion peak at m/z 286 that depict a molecular formula of C20H14O6. The compound has a molecular mass of 330.3 g/mol. The compound 1 was isolated as a yellow solid, its mass spectral data (1H-, NMR and LC-MS) are consistent with literature data for 3, 4', 7, 8'-tetrahydroxyflavone.

Table 1: Primary screening of extract/fractions of A. auriculiformis against 14 disease-related protein kinases#.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cdk2</th>
<th>Cdk5</th>
<th>Cdk9</th>
<th>CLK1</th>
<th>GSK3</th>
<th>Dyrk1A</th>
<th>PIM1</th>
<th>Haspine</th>
<th>RIPK3</th>
<th>mCLK1</th>
<th>LdTLK</th>
<th>CK1</th>
<th>AuroraB</th>
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<tbody>
<tr>
<td>CHCl3</td>
<td>110</td>
<td>106</td>
<td>105</td>
<td>70</td>
<td>65</td>
<td>91</td>
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<td>113</td>
<td>84</td>
<td>96</td>
<td>73</td>
<td>116</td>
</tr>
<tr>
<td>ETOAc</td>
<td>10.0</td>
<td>13.0</td>
<td>9.0</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
<td>2.0</td>
<td>2.0</td>
<td>31.0</td>
<td>28.0</td>
<td>52.0</td>
<td>-</td>
<td>30.0</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>39.0</td>
<td>39.0</td>
<td>14.0</td>
<td>21.0</td>
<td>-</td>
<td>2.0</td>
<td>6.0</td>
<td>19.0</td>
<td>48.0</td>
<td>45.0</td>
<td>-</td>
<td>-</td>
<td>12.0</td>
</tr>
<tr>
<td>Compound 1</td>
<td>-</td>
<td>29.0</td>
<td>14.0</td>
<td>16.0</td>
<td>32.0</td>
<td>11.0</td>
<td>10.0</td>
<td>24.0</td>
<td>-</td>
<td>22.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

#Indicates the results of the primary screening performed using 50 µg/ml of the extract, fractions and isolated compound. Data are expressed in % maximal activity i.e measured in the absence of the inhibitor. ATP concentration used in the kinase assays was 15 µmol/L. (values are means, n=2). Kinases are from human origin unless specified: Ssc, Sus scrofa; Rn, Rattus norvegicus; Mm, Mus musculus; Pf, Plasmodium falciparum; Lm, Leishmania major; Ld, Leishmania donovani. (-) not tested

Table 2: Effect of extract/fractions on Kinase Inhibitory Activity of A. auriculiformis# (IC50) µg/mL.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cdk2</th>
<th>Cdk5</th>
<th>Cdk9</th>
<th>CLK1</th>
<th>GSK3</th>
<th>Dyrk1A</th>
<th>PIM1</th>
<th>Haspine</th>
<th>RIPK3</th>
<th>mCLK1</th>
<th>LdTLK</th>
<th>CK1</th>
<th>HsAuroraB</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETOAc</td>
<td>7.5</td>
<td>6.0</td>
<td>4.5</td>
<td>-</td>
<td>9.0</td>
<td>-</td>
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<tr>
<td>n-Butanol</td>
<td>45.0</td>
<td>52.0</td>
<td>17.5</td>
<td>-</td>
<td>15.0</td>
<td>-</td>
<td>20.0</td>
<td>1.3</td>
<td>17.5</td>
<td>25.0</td>
<td>5.0</td>
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<td></td>
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<tr>
<td>Compound 1</td>
<td>-</td>
<td>&gt;50</td>
<td>2.28</td>
<td>9.99</td>
<td>8.45</td>
<td>2.05</td>
<td>30.1</td>
<td>&gt;50</td>
<td>-</td>
<td>25.0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#Values reported in the are a mean of n=3 independent experiments. ATP concentration used in the kinase assays was 15 µmol/L. Kinases are from human origin unless specified: Ssc, Sus Scrofa; Rn, Rattus norvegicus; Mm, Mus musculus; Pf, Plasmodium falciparum; Lm, Leishmania major; Ld, Leishmania donovani. (-) not tested.
C_{13}H_{16}O_{5}, suggestive of a mono flavonoid nucleus (Figure 1). The proton NMR revealed a tetrahydroxy substituted flavonoid nucleus showing a monosubstituted ring B and a 7,8-dihydroxy substituted ring A flavonoid nucleus respectively, this depict compound 1 to be 3,4',7,8-tetrahydroxy flavone (Figure 1); the spectra compares well with literature.\(^\text{17}\) This is the first report of this flavonoid from Acacia auriculiformis. This compound has been isolated previously from Acacia rhodoxylon.\(^\text{18}\) Primary screening of the isolated compound against eight disease related protein kinases (Table 1) indicates that the compound inhibited more than 50% activity of all the kinases, for example against DYRK1A and PIM 1 kinases, the compound inhibits 89% and 90% total kinase activity respectively. Table 2 shows the IC_{50} values for compound 1 when tested over a wide range concentration from 0.016 to 50 μg/ml. The table reports the inhibitory activity of the compound against the mentioned kinases. The compound was active against six out of 8 kinases tested; however, the compound was found to be inactive against Haspine and CDK5 kinases, the IC_{50} values of the compound against DYRKIA and CDK9 was estimated at 2.05 and 2.28 μg/ml respectively indicating that the isolated flavonoid is a DYRK1A and CDK9 inhibitor. Flavonoids have been shown to have the ability to modify or modulate the activities of some enzymes involved in cell surface signal transduction, immune function and transformation, tumor growth and metastasis.\(^\text{19,20}\) The inhibitory effect of flavonoids in growth of malignant cell could be a consequence of their interference with protein kinase activities involved in the regulation of cellular proliferation and apoptosis.\(^\text{21}\)

**CONCLUSION**

The ethylacetate of fraction of Acacia auriculiformis was shown to be the most active among the three extract/fractions investigated for protein kinase inhibitory activity and from the ethylacetate fraction, 3, 4', 7, 8-tetrahydroxyflavone was isolated and was found to be a DYRK1A and CDK9 inhibitor. This result might justify the cancer and inflammation potentials of this plant.

**ACKNOWLEDGEMENT**

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**CONFLICT OF INTEREST**

The authors declared no conflict of interest associated with this work.

**ABBREVIATIONS**

CD3OD: Deutirated methanol; DMSO: Dimethyl Sulphoxide; LC-MS: Liquid Chromatography-Mass Spectrometry; MCI: MilliCurie; MS: Mass Spectrometry; NMR: Nuclear Magnetic Resonance; TLC: Thin Layer Chromatography.

**REFERENCES**

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SUMMARY

• The Chloroform extract, ethylacetate and n-butanol soluble fractions of the stem bark of Acacia auriculiformis A. Cunn. ex Benth. were screened for primary activity against disease related protein kinases. The Chloroform extract was found to be inactive, while both the ethylacetate and n-butanol soluble fractions inhibited all the kinases tested. The \( IC_{50} \) for both the ethylacetate and n-butanol fractions were also investigated. The ethylacetate fraction exhibited the highest activity against Haspine kinase with \( IC_{50} \) of 1.0\( \mu \)g/ml, while the n-butanol also gave the highest activity against Haspine kinase with \( IC_{50} \) of 1.3\( \mu \)g/ml. From the ethylacetate fractions, 3', 4', 7', 8-tetrahydroxyflavone was isolated. The compound exhibited the maximal activity against DYRK1A kinase with an \( IC_{50} \) of 2.05 \( \mu \)g/ml followed by CDK9 with \( IC_{50} \) of 2.28 \( \mu \)g/ml. 3', 4', 7', 8-tetrahydroxy flavone isolated for the first time from this plant was found to be a DYRK1A and CDK9 inhibitor which might justify the anticancer potentials of this plant.

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