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Phytochemical screening and identification of compounds in the leaves of *Callitris glauca* (Cupresseceae)

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ABSTRACT

Phytochemical screening of the acetone extract of the leave of Callitris glauca indicate the presence of flavonoids, glycoside, saponin free reducing sugar however alkaloid are absence in this specie. The characteristic shades of fully methylated biflavonoids in U.V light has been found to provide a means for quick and satisfactory identification of biflavonoid, hence three biflavonoids, amentoflavones, cupressuflavones and hinokiflavones were identified. The glycoside after hydrolysis afforded quercetin as the genin and two sugars were identified as rhamnose and glucose.

Keywords: Callitris glauca ,chromatograph , flavones and glycosides

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INTRODUCTION

During the past century, the extra ordinary results of research have unquestionably led to success at an exponential rate, which the practitioner of modern medicine now enjoys. However the exclusive use of this research oriented approach with little regard for knowledge acquired through the empirical method has served to delay the application of many potential benefits [1].

World health organization has estimated that perhaps about 80% of more than 4000 million people on earth rely chiefly on traditional medicine for their primary health care needs [2]., and also can safely be presumed that a major part of traditional therapy involve the use of plant extract or their active principles. Such treatments include the administration of infusion boiled parts, as some of the natural drugs are not usually in the form of tablet or pills [3].. Although the structure of some plant constituents are now known, many compounds that remain undiscovered in plant are beyond the imagination of scientist. [4].. The chemical constituents in medicinal plant usually explain the rational for the use of the plants in traditional medicine. [5].

Callitris glauca is a shrub or slow growing tree, eventually coming up to 80 feet high with hard furrowed, greenish brown bark. Leafy branches divided into fine bushy sprays. Leaves about $^{1}/_{10}$ inch long commonly glaucous, dorsal surface rounded giving the branchlets texted, non-ribbed appearance, Cone solitary or in clusters, globase, up to $^{5}/_{8}$ inch, in diameter, on stalk about $^{1}/_{3}$ inch long. Cone scale 6, varying in size on the same scaled, small ones half to three quarters the size of the larger, woody but thin, separating almost to the base, when the cone opens never tubeculate seed reddish brown with 2-3 broad pale wings [6].

The traditional use of Callitris glauca has not yet been recorded but that of Callitris columellaris of the same family has been recorded.

Callitris columellaris of which part was not specified has been used in Australia as salves (oil) or washes for the treatment of cold or chest infection [7].

Callitris hugelli of the same family has been reported to have essential oil of antimicrobial activity. It was shown to have perfume flavour. Ten essential oils were tested for antimicrobial activity of which the Australian sandalwood was most active against staphylococcus aureuis and candid albicans. Pseudomonas aeruginosa was the most resistant microorganism tested. [8].

The biological activities for the extract of Callitris columellaris only have been recorded. The ethanol (95%) extract of leaf and stem of Callitris columellaris after cell culture was found to have cytotoxic activity active at 0.4 micro gram per ml (0.4mcg /mL) [9].

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MATERIALS AND METHODS

Extraction:

Dried and coarsely powdered leaves of Callitris glauca (150g) were refluxed with petroleum ether (60-80^{o}C) for 10 hours. The extract was decanted off and fresh quantity of the petroleum ether was added again and refluxed for another 10 hours.

The defatted leaves were completely dried and extracted with acetone. The combine acetone extracts where concentrated on water bath whereby a highly viscous greenish – brown mass was obtained. This was refluxed with petroleum ether (60-80^{o}C), benzene and chloroform successively until the solvent in each case was almost colourless. The residue left behind was then treated with hot water. The water insoluble portion was dissolved in acetone and dried under reduced pressure. A solid brown residue (12.8g) obtained respond to usual flavonoid colour tests (table 3) was marked 'A'

Table 3. Colour test of fraction 'A'

Reagent	Colour produced	Inference
(1) Mg- HCl	Orange	++
(2) Alc.Ferric Chloride	Dark green	+ + +
(3) Zn-HCl	Red	+ +

The aqueous solution was extracted with ethyl acetate. The process was repeated twice. . The ethyl acetate extract where combined and the solvent was recovered under reduced pressure. The semi- solid residue was marked 'A_1' and respond to usual flavonoidal test (table 4)

Table 4. Colour test of fraction 'A_1'

Reagent	Colour produced	Infrence
(1) Mg- HCl	Orange	++
(2) Alc.Ferric Chloride	Dark green	+ + +
(3) Zn-HCl	Red	+ +

Thin layer chromatographic examination of fraction 'A':

Thin layer chromatographic plate (5x20cm) 0.5mm thickness was prepared by usual method using silica gel G (E. Merck). The sample of 0.1% of 'A' was dissolve in alcohol and spotted manually using a capillary tube. The plate was developed in benzene: pyridine: formic acid (36:9:5) BPF as solvent system. After development, on examination of the chromatogram under U.V. light and then sprayed with ferric chloride the presence of one spot with trailing was revealed fig 1 this was purified with column chromatography

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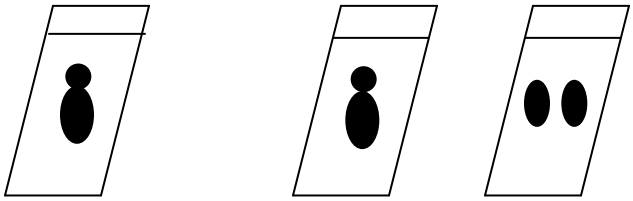
Fig. 1: TLC chromatogram of fraction 'a' in three different solvent systems (BPF, 36:9:5;TPA, 10:1:1 and B:EA:A. 8:5:2) respectively



Purification of fraction 'A' by column chromatography:

A well-stirred suspension of silica gel (120 mesh) (E. merck) 150g in petroleum ether (60 – 80°) was poured into a column (120cm long and 50mm in diameter) when the absorbent is well settled, the excess petroleum ether was allowed to pass through the column (drained out). The extract 'A' (2g) was dissolved in acetone and was added to the column. After development of the column a circular filter paper was placed on the top of the absorbent. The column was eluted with organic solvents in increasing order of polarity as shown in (table 5). The organic solvent include (a) petroleum ether (b) chloroform, (c) ethyl acetate and then washed with (d) ethyl acetate and methanol in the ratio 9:1. On confirming that portions (b,c and d) are the same on T.L.C they were combined and evaporated to dryness and labeled fraction 'A_2'. This responded to usual colour test for flavonoid. (table 6). Thin layers chromotography (table 7) and paper chromotography (table 8) examination of fraction 'A_2' in different solvent system showed the present of 3 spots fig 2 (table 9).

Table 5. Nature of product from coloumn chromatography for fraction 'A'

Solvent	Nature of product
(1) Pet-ether (60-80°C)	Greenish gummy mass
(2) Chloroform	Brownish solid
(3) Ethyl acetate	Brownish solid
(4) EA: MeOH (9:1)	Brownish solid

Table 6. Colour test of fraction 'A_2'

Portion	Reagent			Inference
	Mg-HCl	Alc.$FeCl_3$	Zn- HCl	
(1) Pet-ether	-	-	-	-
(2) Chloroform	Orange	Dark green	Red	+ + +
(3) Ethyl acetate	Orange	Dark green	Red	+ +
(4) EA: MeOH (9:1)	Orange	Dark green	Red	+ +

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Table 7. Solvent system for tlc of fraction 'A_2' and r_f values of spots developed

Solvent system	R_f values				
Benzene: pyridine: acetic acid (BPF)	36:9:5	0.17	0.32	0.52	0.57
Toluene: pyridine: acetic acid (TPA)	10:1:1	0.07	0.13	0.40	0.33
Benzene: ethyl acetate: acetic acid (B: EA: A)	8:5:2	0.22	0.35	0.67	0.73

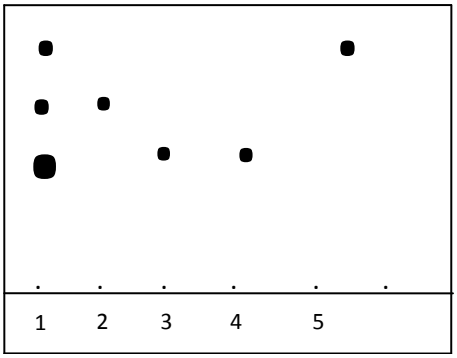
Table 8. Paper chromatographic examination of fraction 'A_2'

Solvent system	R_f values
Butanol: acetic acid: water (BAW) (4:1:5)	0.40
Acetic acid: water (6: 4)	0.32
Benzene: pyridine: acetic acid (100:1:100)	0.55
Ethyl acetate: formic acid: water (10:2:3)	0.53

Table 9. R_f value of spot in T.L.C Chromatogram of fraction 'A_2'

SPOTS	R_f value
1 = fraction 'A_2' from column	0.17, 0.32, 0.57.
2 = authentic hinokiflavone	0.32
3 = authentic amentoflavone	0.17
4 = authentic cupressuflavone	0.17
5 = authentic quercetine	0.57

Fig.2 TLC chromatogram of fraction 'A_2'



Methylation:

The fraction 'A_2' (2mg), potassium carbonate (2g) and dimethyl sulphate (1ml), in dry acetone (200ml) was refluxed on water bath for about 8 hours. It was filtered and the residue washed several time with hot acetone. The filtrate and washings were combined and evaporated to dryness. The yellow residue thus obtained washed 2-3 times with petroleum ether and then taken up in chloroform (100ml) and washed several times with water in a separating funnel. The chloroform solution was concentrated and marked fraction 'A_3' and this does not respond to usual flavonoid colour test (table 10)

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Table 10. Colour test of fraction 'A_3'

Reagent	Colour produced	Inference
(1) Mg- HCl	No colour change	-
(2) Alc.Ferric Chloride	No colour change	-
(3) Zn-HCl	No colour change	-

Thin layer chromatography of fraction 'A_3':

Fraction 'A_3' was spotted with authentic permethylated compound in BPF (36: 9: 5) and 3 spots (fig 3) where observed which correspond to R_f values equivalent to those of authentic Amentoflavone Hexamethylether; Cupressuflavone Hexamethylether; and Hinokiflavone pentamethylether (table 11). TLC behaviour of fraction 'A_3' using BPF (36:9:5) as viewed under U.V light and as developed in iodine vapour (table 12) showed the spot with different fluorescence.

Fig.3 TLC chromatogram of methylated portion fraction 'A_3'

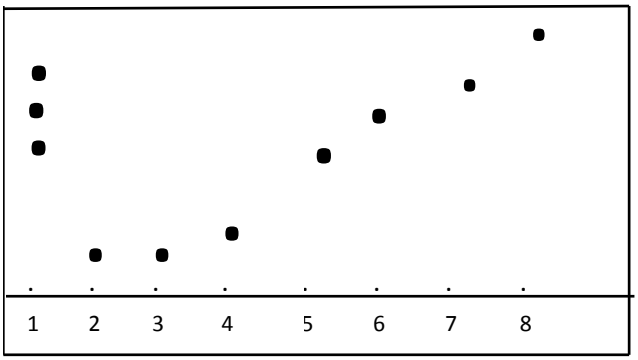

Table 11. R_f value of spot in TLC chromatogram of fraction 'A_3'

Spots	R_f value
1 = Methylated portion	0.40, 0.43, 0.52
2 = Amentoflavone	0.17
3 = Cupressuflavone	0.17
4= Hinokiflavone	0. 32
5= Amentoflavone hexamethylether	0.40
6= Cupressuflavone hexamethylether	0.43
7= Hinokiflavone pentamethylether	0.52
8 = Quercetine	0.57

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Table 12. TLC behaviour of Callitris glauca constituents in fraction 'A$_3$'

Spot	Colour under U.V light	Iodine vapour	R_f value
1 = Amentoflavone hexamethylether	Bright yellow	Dark yellow or light brown	0.40
		Light yellow	
2 = Cupressuflavone hexamethylether	Light yellow	Light yellow	0.43
3 = Hinokiflavone pentamethylether	Faint yellowish blue		0.52

Fraction 'A$_1$':

The acetone solution of fraction 'A$_1$' was subjected to paper chromatographic analysis using Whatmann paper no.1. In each solvent system, the chromatograms were developed for 10 hours. After drying in fume cupboard, the chromatograms were examined under U.V. Light, which revealed the presence of two spots with trailing.

Hydrolysis of fraction 'A$_1$':

10mg of fraction 'A$_1$' was dissolved in water and hydrolyzed by refluxing with 2ml of 0.6M hydrochloric acid. The hydrolysis appeared to be completed within a few minutes, but the heating was continued for 2 hours to ensure complete hydrolysis. After leaving over-night, the aglycone was filtered, washed, dried at room temperature and marked fraction B.

Chromatographic identification of the sugar:

The filtrate (from which the aglycone was removed) was neutralized with aqueous ammonia until it was neutral to litmus paper. It was concentrated under reduced pressure to a syrupy mass. This syrupy mass was chromatographed on Whatmann paper No. 1 using n-Butanol: Acetic acid: Water. (4:1:5) as solvent system with authentic (reference sugar) samples (table 13). The chromatogram after development was dried in a fume cupboard sprayed with aniline – H – phthalate reagent and then heated at 110^{o}C for 5minutes. After development on examination the chromatogram shows the presence of two spots equivalent to R_f values of authentic glucose and rhamnose (fig 4).

Table 13. R_f value of spot in paper chromatogram of fraction 'A'

1 = Hydrolyzed Portion B
2 = Arabinose
3 = Xylose
4 = Glucose
5 = Galactose
6 = Rhamnose
7 = Fructose
8 = Free sugar A$_1$

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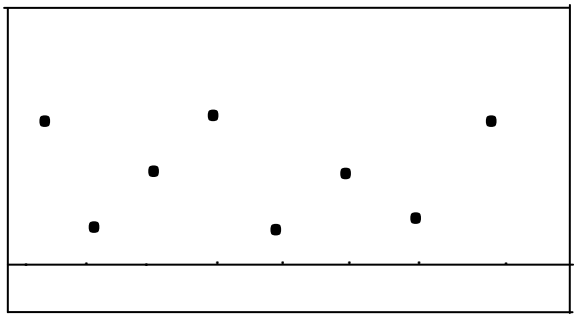
Fig. 4.: Paper chromatogram of fraction 'A' after hydrolysis



Preparation of authentic sugar:

The authentic sugar rhamnose, glucose, galactose, fructose, xylose and arabinose were prepared by dissolving small sample of the sugar in distilled water.

Quantitative evaluation of Callitris glauca leaves:

(a) Determination of total ash value
(b) Determination of acid insoluble ash value
(c)Determination of water-soluble ash value
(d)Alcohol soluble extractive value
(e)Water soluble extractive value [10]

RESULTS

Phytochemical screening:

The result of the phytochemical screening of fraction 'A' was summarized in table 1

Table. 1 Phytochemical Screening Result

Group	Test	Inference
FLAVONOIDS	(a) Ferric chloride test	+ + +
	(b) Lead acetate test	+
	(c) NaOH test	+ + +
	(d) Shinoda test	+ +
CARBOHYDRATE	General test	-
	Test for starch	+
	Barfoed's test for sugar	+
	Fehling's test for reducing sugars	+ +

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	Combined reducing sugar	+
CARDIAC GLYCOSIDE	Liebermann-Burchard test	+
	Salkowskii test	+
	Cardenolide	++
	Keller – Killiani Test	+
SAPONIN	Frothing test	+ + +
TANINS	General test	+
	Phlonatanins test	-
	Anthracene derivatives	-
ALKALOIDS	General tests: Wagner, Dragendoff's & Mayer's reagents	-
	Morphine alkaloid (Radulescu test)	-
	Indole alkaloid (Extract+conc. H_2SO_4+ $K_2Cr_2O_7$)	-
	Quinoline alkaloid (Thalleiqiune test)	-
	Tropane alkaloid (Vitali – Morin test)	-

KEY Faintly present = +; Moderately present = ++; Highly present = +++; Absent = -

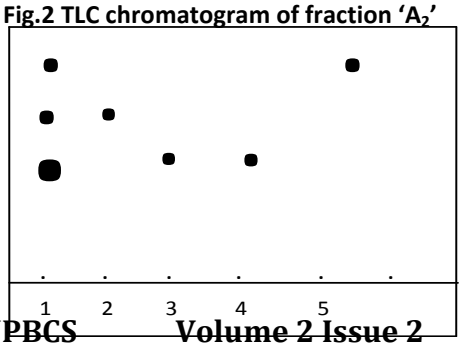
DISCUSSION

The plants acetone extract was extracted with various organic solvents to afforded a dark brown mass 12.8g The mass gave dark green colour with alcoholic ferric chloride, orange colour with Mg-HCl and red colour with Zn-HCl. This clearly indicates that this fraction contain flavone nucleus. The extract was treated with hot water and filtered. The hot water insoluble portion afforded a brown residue, which gave various colour tests for flavonoid and was marked 'A' table 3 The hot water soluble portion was extracted with ethyl acetate to afford a dark brown mass which gave various colour test for flavonoid and was marked 'A_1'table 4 Fraction 'A' after treatment with pet ether, chloroform and benzene was passed through column of silica gel and eluted with various solvent of varying polarity. The result of the column chromatography was summarized in table 5. The eluate collected from chloroform, ethyl acetate and washing showed similar behaviour on T.L.C. table 6 were combined and marked fraction 'A_2'.

Flavonoid are primary coloured pigment and moderately polar compounds due to the presence of various hydroxyl groups on the nucleus. The fraction also gave positive colour test for flavonoid table 7

The fraction 'A_2' was chromatographed in several solvent system, table 8 Fraction 'A_2' revealed the presence of 3 distinct spots in several solvent system fig 2 table 9 Solvent systems include benzene: pyridine: formic acid (36:9:5)

Fig.2 TLC chromatogram of fraction 'A_2'



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Acid hydrolysis of fraction 'A' yielded an aglycone. The fraction was co-chromatographed on whatmann paper No.1, with two solvent systems, against authentic samples of aglycone table15 and where identified as quercetin.

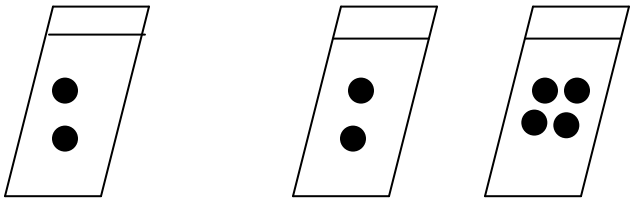
Table 14: Solvent system development

Toluene: pyridine: acetic acid	10:1:1
Toluene: acetone	19:1
Benzene: ethyl acetate: acetic acid	8:5:2
Ethyl acetate: formic acid: water	10:2:3
Chloroform: acetone: water	1:2:1
Ethyl acetate: methanol: water	10:14:1
Methanol: acetic acid	9:1

Table 15: Solvent system for paper chromatogram of aglycone before hydrolysis of fraction 'A'

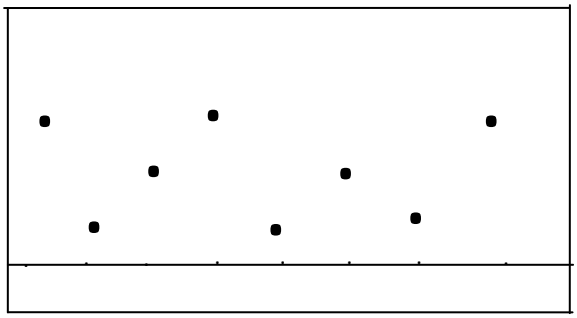
n-butanol: acetic acid: water	4:1:5
Benzene : pyridine: n-butanol : water	1.3.5.3

Fig.5 Paper chromatogram of aglycone before hydrolysis



The aqueous portion after neutralization gave a positive test for reducing sugars. This fraction was co-chromatographed on whatmann paper No.1 with reference sugar sample in different solvent system for 18 hours fig 4. The sugar portion gave the same R_f value table 13 with Glucose and Rhamnose in two different solvent system table 15

FIG. 4.: Paper chromatogram of fraction 'A' after hydrolysis



Identification of biflavonoids:

All biflavonyls except morelloflavone and those of GB series are derived from apigenin with a C-C or C-O-C interflavonyl linkage. It is well known that if there are several substituents in the same molecule, the effect of each substituent on the absorption affinity is very approximately additive.

Amentoflavone and cupressuflavone having an equal number of phenolic hydroxyls would be expected to show the same R_f values. They are so close, that they defy identification and separation in case where they occur together in the same plant. The small difference in the R_f values in BPF of amentoflavone and cupressuflavone may, however, be explained by, their relative departure from planarity with subsequent variations in the magnitude of the conjugative effect.

Hinokiflavone with five free phenolic hydroxyls and the sixth involved in an ether type interflavonoid linkage showed an R_f of 0.43 (BPF). This is much higher than those of the two biflavonyl type biflavones mentioned earlier with R_f of 0.40.

The absorption affinity differences index with increasing methylation so much so that fully methylated biflavone involving various modes of interflavonyl linkage were found to show sizeable difference in R_f values table 11 The different shades of the spots of these fully methylated derivatives in U.V. light (BPF) and as developed in iodine vapour table 12 were also found to be of some help in their identification.

BPF thus have been established as excellent developing system for the separation and identification of fully methylated biflavones.

Table 2. Determination of ash value of Callitris glauca leaves

Total ash value content	9.833(%)
Acid insoluble ash value	5.7(%)
Water soluble ash value	1.17(%)
Alcohol soluble extractive value	1.17(%)
Water extractive value	5.2(%)

The quantitative evaluation of the dry powdered leave (table 2) gave a total ash value 9.8%, acid insoluble ash value 5.7%, water soluble ash value 1.17%, alcohol soluble extractive value 1.17%, and water extractive value 5.2%

CONCLUSION

It has been concluded that the leaf of Callitris glauca (Cupresseceae) show the presence of a flavone (quercetin), two sugars as glucose and rhamnose and three biflavonoid as amentoflavone, Hinokiflavone and cupressuflavone.

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REFERENCES

[1] Dastur, J. (1964): Useful Plants of India and Pakistan. A popular hand book of trees and plants of industrial economics. D.B. Taraporevera India Pp 185
[2] Akerele (1990): Medical Plant and Primary Health Care. An agenda for action in drugs monitor 10. 8-9
[3] Oliver, B.(1960): Nigerian useful plants part 11 the Nigerian field Longman Nigeria 24 Pp104
[4] Sievers, A.F.(1930): American medicinal plants of commercial importance USDA. Miscellaneous Pp150
[5] Farnsworth NR. Journal of Pharmacy 1984;38:1-13
[6] Chamberlien, C.J. (1965): gymnosperm, structure and evolution, Johnson, reprint Co. N.Y.,U.S.A. Pp87
[7] O'Connel JF. (1983):economic botany 391 Pp80-81
[8] Givandan, S.A. and Gilmas,G. (1979): Internation journal of pharmacy 1979;4(2): 23-5
[9] Aynechi Y. Journal of Pharmaceutical Science 1971;60:121-2
[10] British Pharmacopoeia (1993): vol II: A157; Appendix XIM.