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# Identification and Structure Elucidation of Principle Active Flavonoids from Aqueous-Methanolic Extract Of *Olea Cuspidata*

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***Abstract:*** *Olea cuspidata, a subspecies of Olea europaea and native to Indian Himalaya, is a oil producing evergreen traditional medicinal plant. Olea cuspidata is an excellent fodder producing plant and its leaves have been used as a good supplement to lactating animals of Kumaun hills. The leaves of the plant have medicine to cure cardiovascular and various forms of inflammatory diseases by the local inhabitants of kumaun hills. Therefore, various extracts derived from the leaves of the plant were screened for the isolation and characterization of prominent active catechol grouping flavonoids.*

***Keywords:*** *Olea cuspidata, flavonoids, antioxidant activity.*

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## I. INTRODUCTION

Flavonoids, a group of naturally occurring polyphenolic heterocyclic secondary metabolites and biogenetically derived from phenyl propanoids, have been identified as a prominent antioxidant and radical scavenging activities (Jovanovic et al.1994; Bors et al; 1990; Pietta, 2000). A high intake of dietary flavonoids from fruits, vegetables and herble extracts, inhibit the intial processes of diseases associated to oxidative stress. It has been established that the curing of many diseases from the extract of traditional medicinal plants have been attributed to the presence of prominent antioxidative polyphenolic compounds. Flavonoids have been identified as potent antioxidant, anti-inflammatory and antiviral (Kinghorn et. al.; 2004 ). *Olea cuspidata* , a subspecies of *Olea europaea* and native to Indian Himalaya, is a oil producing evergreen traditional medicinal plant. *Olea cuspidata* is an excellent fodder producing plant and its leaves have been used as a good supplement to lactating animals of Kumaun hills. The leaves of the plant have medicine to cure cardiovascular and various forms of inflammatory diseases by the local inhabitants of kumaun hills. Therefore, various extracts derived from the leaves of the plant were screened for the antioxidant activity and isolation and characterization of prominent active catechol grouping flavonoidds.

## II. MATERIALS AND METHODS

### (I) Plant materials collection, identification and preparation

Plant materialwere collected from NCC ground Almora. The authentication of the species was made by Prof. P.C.Pandey, Department of Botany , Kumaun University, S.S.J. Campus Almora and Voucher Specimen no. (9) has been deposited in Botany department, Kumaun University, S.S.J. Campus Almora.

### (II) Extraction and isolation of flavonoids:

3 kg air dried and powdered leaves of *Olea cuspidata* was extracted with 70% aqueous-methanol by cold percolation method for six days. The aqueous-ethanolic extract was reduced in vacuo until only water layer(approx. 150 ml) remained. It was partitioned with $CH_2Cl_2$ and n-BuOH . The BuOH fraction evaporated to dryness and residue was dissolved in 70% aq. EtOH. It was chromatogramed on Whatmann. NO. 3 PC (15 sheets) using 30 % HOAc as a developing solvent. An unresolved broad continuous horizontal dull purple/violet fluorescent band was inspected with light. It was cut and eluted with 50% EtOH. The aqueous ethanolic elute was evaporated to dryness at 40ºC in Rota-evaporator. The residue was chromatographed on Cellulose CC. It was eluted initially with $H_2O$ and then increasing polarity with acetic acid. After two times repeated development , a broad dark purple/violet fluorescent band was observed on between faster moving violet and slower moving dull yellow band with UV light. Each band was eluted separately. The aqueous-AcOH elute of faster moving and slower moving band, representing fraction - 01 and 02 respectively were collected separately by monitoring the CC with UV light. The flavonoidal compounds, (A) and (B) were finally isolated from above fraction by RPPC and Sephadex LH-20 column as follows:

## III. RESULTS AND DISCUSSION

### 3.1 Structural Elucidation Of Compound (A)

The compound (A) appeared as a dark purple or violet spot on PC under UV light and changed to yellow-green with $NH_3$ vapours, indicating the presence of 4', 5-hydroxyl groups in A-ring of flavone (Mabry et al., l970; Markham, 1989). When cellulose TLC of the compound was sprayed with NA reagent. The spot turned to orange indicating the presence of ortho-dihydroxyl group in the B-ring (Mabry et al., 1970: Markham. 1976; Geiger and Homberg 1983; Homberg and Geiger, 1980 ). When PC of the compound was sprayed with alcoholic solution of $ZrOCl_2$, it turned to fluorescent yellow-green indicating the presence of free 5-OH group in A-ring (Feigl, 1960). The yellow-green fluorescence of the compound changed to yellow when the compound is treated with citric acid (2% aq. Sol.) indicating the 3-OH group of the compound is substituted. The methanolic solution of the compound did not produce colour with vanilline + HCl, indicating absence of free 7-OH group (Hillis and Urbach, 1958). Thus on the basis of colour reactions, the compound (A) has free hydroxyls at C'-3', C-' and C-5. The compound (A) gave positive Molisch and Feigl test for sugar indicating the glycosidic nature of the compound (Feigl 1960). On the basis of Rf values of compound on PC with various solvent systems. $H_2O$, 15% HOAc. 30% HOAc, 50% HOAc BAW and BEW Indicated a flavonol disaccharide but not a flavonol-3-O-oligosaccharide compound. (Markham, 1982; Harborne and Williams. 1975, Markham, 1989; Mabry et al., 1970).Compound (A) be identified as a probably Quercetin-3-7-diglycoside .On complete acid hydrolysis with 2N HCl at 100ºC for 45 min. It gave an aglyconc and sugars, glucose and rhamnose.

The aglycone crystallized as pale yellow needles from ethylacetate- petroleum ether mixture, m.p. 315-18º and analysed for $C_{15}H_{10}O_7$. The MS of aglycone exhibited a molecular ion at m/e 302(100%), 273 (aglycone CO-H) 153 (A + H) and 137 (B2+). The aglycone appeared as a dull yellow fluorescent spot on paper chromatogram under UV light and dull yellow colour remain unchanged in presence of $NH_3$, indicated a flavonol compound with free 5-OH group. When paper chromatogram was sprayed with Naturstoffeagenz A (NA). The spot turned orange indicating on ortho-dihydroxyl group in the B-ring. Compound exhibited UV maxima in MeOH at λmax at 370 nm band (I), λmax at 255 nm band (II) and λ max at 300 nm band (III) and the shifts obtained with diagnostic reagent with $AlCl_3$, $AlCl_3$+HCl, NAOAC, NAOAc + $H_3BO_3$ and $CH_3ONa$ suggested it has free hydroxyls at C-3,C-5, C-7, C-3' and C-4' (Mabry et al., 1970). The 'HNMR (DMSO-d6), showed signals at δ 7.32 (lH, d, J=1.9 Hz), 7.20 (lH, dd, J=8.3 Hz, 2.0 Hz). 6.88 (lH. d. J= 8.3 Hz). 6.40 (lH, d, J=1.9 Hz) and 6.18 (lH, d, J= 1.9 Hz). These signals could be assigned to H-2', H-6', H-5', H-8 and H-6 respectively. On acetylation it gave penta acetate, m.p. 198°C. The aglycone was identified as Quercetin

The EtOAc soluble of neutral acid hydrolysed extract, the Quercetin rich fraction was separated and remaining aqueous layer was repeatedly evaporated to dryness at 90ºC in water bath. The residue was dissolved in EtOH and chromatographed on Whatman No. 1 PC using BAW (n-BuOH- AcOH-$H_2O$, 4:1:5, V/V upper layer) as a developing PC was sprayed with benzidine reagent. It was dried in oven at 110ºC for 10 minutes. Two dull- brown spots of sugars were visualized on PC were identified as a glucose and rhamnose by CoPC with their standards using three solvent systems, BAW, BEW and BPW. Thus, glucose and rhamnose were identified from acid hydrolysed mixture of compound (A). `

$H_2O_2$ oxidation of the compound (A) was made by the standard method of Chandler and Harper (1960). The oxidized product, a bright yellow UV fluorescent compound, was identified as an f1avonol compound with free hydroxyls at C-3', C-4' and C-5. Further the compound was isolated from $H_2O_2$ oxidised mixture by Sephadex LH-20 CC. The $H_2O_2$ oxidised residue was chromatographed on Sephadex LH-20 column using 50% MeOH as an eluent. After inspecting CC with UV light a bright yellow fluorescent band was observed on column. It was eluted and collected separately. The aqueous-methanolic elute was evaporated to dryness and gave a compound representing structure A(a), It gave blue colour with $FeC1_3$, positive tests against α-naphthol and Feigl test. Thus the compound A (a) was identified as 3', 4' 5-trihydroxy-flavonol-glycoside. The compound A(a) was hydrolysed with 2N HCl at 100ºC for 1 hour. The acid hydrolysed mixture was neutralised with $BaCO_3$.The neutral solution was fractionated with EtOAc. The EtOAc soluble gave a f1avonol compound, Quercetin. The sugar rhamnose was identified from its neutral aqueous layer by CoPC with its authentic, using two solvent systems BAW and BEW. Thus, the compound A (a) was identified as Quercetin-7-O-α-L-rhamnoside by CoPC with its authentic. Further, the compound (A) was treated with enzyme, or-rhamnosidiase (Aldrich Chem.) The sugar released after enzymatic hydrolysis was identified as rhamnose. The enzymatic hydrolysed product gave a Quercetin-3-O- glucoside (CoPC). Thus, the enzymatic hydrolysed product was identified as a compound Quercetin-3-O-glucoside, representing structure A (b). Thus, compound (A) Was identified as Quercetin-3-O-β-D-glucoside-7-α-L - rhamnopyranoside.( Fig.-2).

'HNMR (TMS ether in $CCl_4$) of the compound gave five aromatic signals at 6.35, 6.75, 6.86, 7.45 and 7.65, representing H-6 (IH, d,J= 2.0 Hz), H-8 (IH, d, J=2.0 Hz) H-5' (IH, d, J= 7.5 Hz),(H-6') (I H, dd, J=2.0 Hz) respectively. Two anomeric proton signals at δ 5.20 and 5.90, assignable to the anomeric proton of rharnnose and glucose respectively. A 10 proton multiplet was observed between δ 3.0 to 4.0, identified as rharnnose and glucose protons (except

anorneric protons of glucose and rhamnose). A doublet was observed at δ1.20 with J= 1.0 HZ assignable to $CH_3$ protons of $CH_3$ group of rhamnose. 'HNMR of compound is given in table no.-1.

**Structural Elucidation of Compound (B)**

Compound B appeared as a violet fluorescent spot on PC under light and changed to yellow green with $NH_3$ vapours indicating the presence of free 5- and 4' hydroxtyl groups (Mabry et. al., l970). The colour reactions, UV spectra, mass data and 'HNMR spectra of the aglycone of compound (B) indicated that it was quercetin. The acidified aqueous layer was neutralized with basic lead acetate and filtered. The filtrate was treated with $H_2S$ and lead was removed as lead sulphide. After filtration it was evaporated under vacuo and residue was analysed on PC for identification of sugar constituents. The residue was adsorbed on PC and developed in BAW (n-BuOH-AcOH-$H_2O$, 4: l: 5. V/V upper layer). The dried and developed chromatogram was sprayed with Wykes reagent (p-Anisidine-HCl) and heated at l00º for 6 minutes. A dark brown spot visualized on PC was identified as glucose by its CoPC with its standard.

'HNMR spectra of the compound (B), gave three doublets with large coupling constants in aliphatic region, at δ 5.80 (lH, d, J=7.0 Hz, 3-O-glucosido), δ 5.l6 (lH, d, J=7.6 Hz, anomeic proton of 7-O-glucosiC) and at δ 4.67 (2- terminal glucose-H-l, d, J= 7.6 Hz) representing three anomeric protons of three glucose moieties each with β-configuration and pyranose form (Overend, l972; Altona and Haasnoot, 1980). In aromatic region two meta coupled doublets appeared at δ 6.74 (d, lH, J= l.8 Hz) and δ 6.4l(d, lH, J=l.8 Hz) for H-8 and H-6 of A-ring respectively .While the resonance of B-ring protons appeared at δ 7.69 (lH, d,d, J= l.5 and 7% Hz), δ 7.51 (lH, d, J= l.5 Hz) and at δ 6.80 (1H, d, J= 7.5 Hz) for H-6', H-2' and H-5' respectively. 'HNMR spectra of the compound (B) are given in Table No 2.

The presence of glycosylation at the 7-OH group of compound (B) followed from the down field shift of the resonances of H-6 and H-8 proton to δ 6.4l and δ 6.74 respectively (Mabry et. al, l970). The down field shift of two anomeric protons at δ 5.80 and δ 5.l6 revealed that two glucose moieties are directly linked to aglycone and the high field position of third anomeric proton signal at δ 4.67, indicated a inter glycosidic linkage between glucose-glucose (Massiot et. al, l986). The inter-glycosidic linkage was confirmed as glucosyl (l→2) glucoside as evidenced by appearance of resonance of anomeric proton signal of terminal glucose moiety at δ 4.67 whereas in case of glucosyl (l→6) glucoside it appears between δ 3.56- δ 4.62 (Overend, l972; Altona and Haasnoot, l980). The interglycosidic linkage, l→2 between two glucose moieties was further confirmed on the basis of $H_2O_2$ oxidation of compound (B) by the standard method of Chandler and Harper (1961). $H_2O_2$ oxidation of flavonol glycoside release sugar at 3-position.The compound (B) oxidized with 30% $H_2O_2$ in 0.lM $NH_4OH$ at room temperature for three days. The reaction mixture was partitioned between EtOAc and $H_2O$. Both the layers were decanted and concentrated under reduced pressure l DPC examination of the residue of aqueous layer using n- BAW (n-BuOH-AcOH-$H_2O$, 4: l: 5. V/V upper layer)solvent system gave a brown spot after spraying the dried and developed chromatogram with Wykes reagent (3% p-anisidine HCl in n-BuOH solution) (Wykes, l953). It was identified as disaccharide on the basis of Rf values on PC using different solvent system and sugar chromogenic spraying reagents. The sugar was identified as sophorose by CoPC with its standard.

The residue of EtOAc soluble of $H_2O_2$ oxidised extract of compound (B), gave a yellow amorphous compound. It was purified on Sephadex LH-20 column using 60% MeOH as eluting solvent. A fluorescent band on CC was monitored with UV light and collected separately representing compound B (a). Its aqueous MeOH elute was concentrated and purified by PCC using BAW (n-BuOH-AcOH-$H_2O$, 4: l: 5, upper layer) and 30% HOAc as developing solvents.

Compound B (a) gave free hydroxyl substitution at C-3, C-5, C-3' and C4' while its aglycone gave free-hydroxyl substitution at C-3, C-5, C-7, C-3' and C-4'. Thus the only 7-OH is left to be substituted. The attachment of sugar moiety in 7-position of the flavonol was further confirmed by complete methylation of glycoside of the compound B(a) followed by hydrolysis. Complete methylation of the glycoside by Hakomori's method followed by hydrolysis produced 3', 4', 3, 5-tetra-O-methyl 7-OH flavone which was confirmed by CoPC using BAW, t-BAW, BEW, 30% HOAc solvent system. Complete acid hydrolysis of the compound B (a) gave an aglycone. quercetin and sugar, glucose. The enzymatic hydrolysis the compound B (a) with emulsin obtained from almond, gave similar hydrolyse product. which indicated β-linkage of glucose moiety. Thus, the structure of compound B(a) was identified as Quercetin-7-O-glucoside, representing structure**,** which was finally confirmed by CoPC with its authentic.

Thus, the compound (B) was identified as Quercetin-3-O-Sorphoside-7-O-glucoside. (Fig.- 1).

## IV. CONCLUSION

Our study concludes presence of active flavonoids from aqueous methanolic extract of *Olea cuspidata* . The compound Querecetin and its glycoside isolated from *Olea cuspidata* are responsible for antioxidative activity.Further literature survey reported that *Olea* have prominent antioxidant, antimicrobial, anticancer, antiviral and anti-inflammatory activity, this further supported the use of the plant in the local traditional

medicinal system. Due to the presence of antioxidant the plant material was used in food, medicine and cosmetic industry.

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**Table No. 1-** 'HNMR OF COMPOUND (A) (TMS Ether, in $CCl_4$)

| S.No. | Shift(δ) | Multiplicity, J=Hz | Proton Identification |
|---|---|---|---|
| 1 | 6.35 | 1H,d,J=2.0 Hz | H-6 |
| 2 | 6.75 | 1H,d,J=2.0 Hz | H-8 |
| 3 | 6.86 | 1H,d,J=75 Hz | H-5 |
| 4 | 7.45 | 1H,dd,J=7.5 and 2.0 Hz | H-6 |
| 5 | 7.65 | 1H,d,J=2.0 Hz | H-2' |
| 6 | 5.20 | 1H,d,J=1.5 Hz | H-1" ill shaped rhamnose anomeric proton |
| 7 | 5.90 | 1H,d,J=5.0 Hz | Broad H-1'" glucose anomeric proton |
| 8 | 3.0-4.0 | M, 10 H , for rhamnose+ glucosyl proton | Rest 10 proton glucose+rhamnose |
| 9 | 1.20 | 1H,d,J=1.0 Hz | Rhamnosyl $CH_3$ |

**TABLE NO 2** 'HNMR SPECTROSCOPIC DATA OF THE COMPOUND (B)

| Shift(δ) | Multiplicity(J) | Proton Identified |
|---|---|---|
| 7.69 | 1H, dd, J=1.5 and 7.5 | H-6' |
| 7.51 | 1H, d, J=1.5 | H-2' |
| 6.80 | 1H, d, J=7.5 | H-5' |
| 6.74 | 1H, d, J=1.8 | H-8 |
| 6.41 | 1H, d, J=1.8 | H-6 |
| 5.80 | 1H, d, J=7.0 | 3-glucose-H-1 |
| 5.16 | 1H, d, J=7.6 | 7-glucose-H-1 |
| 4.67 | 1H, d, J=7.6 | 2-terminal-glucose-H-1 |
| 3.0-4.2 | M(broad) | Other protons except protons of anomeric three glucose moieties. |

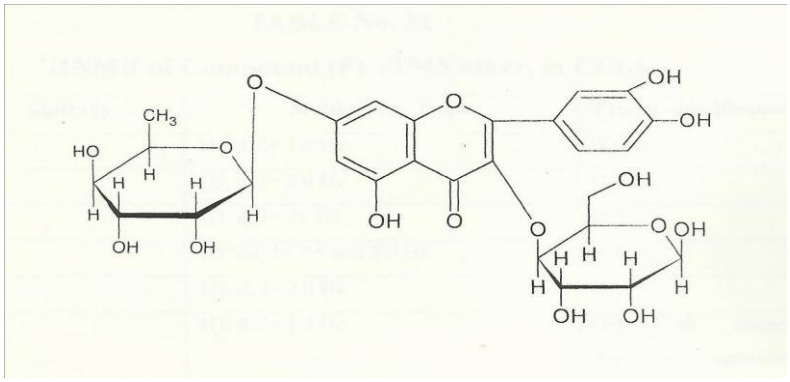

**Fig no- 1-** Compound A

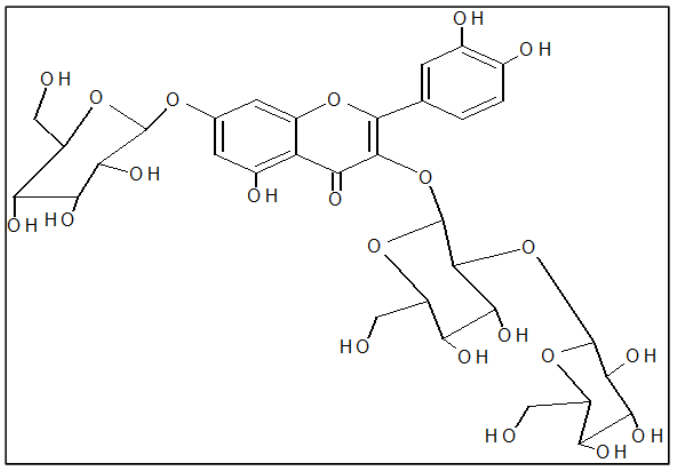

**Fig no- 2-** Compound B

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