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Isolation, Characterization and Pharmacological Studies of A Flavonol Glucoside From *Trichilia connaroides* (W.&A.) Bentilizen

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ABSTRACT

A flavonol glucoside, namely populnin (Kaempferol-7-O-glucoside) was isolated from the leaves of *Trichilia connaroides* (W.&A.) Bentilizen. Investigation of the methanolic leaf extract of *Trichilia connaroides* for analgesic activity led to the isolation of populnin. Structural elucidation of the isolated compound was achieved by IR, ^{1}H-NMR and LC-MS. The methanolic extract and the isolated compound were tested for analgesic activity using the Eddy's hot plate method and the Acetic acid induced writhing test. In both tests populnin showed significant analgesic activity ($P<0.001$) at a dose of 50 mg/Kg b.w. The methanolic extract and populnin did not show any acute toxicity. Isolation of the flavonol glucoside and the activity observed in this study may explain in part the use of the tree in traditional medicine as a crude analgesic drug.

Keywords: *Trichilia connaroides*, Populnin, Methanolic extract, Analgesic activity, Flavonol glucoside.

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INTRODUCTION

Herbal medicine is the oldest form of healthcare known to mankind. Herbs have been used by all cultures throughout history. It was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. The Meliaceae or the Mahogany family is a flowering plant family of mostly trees and shrubs (and a few herbaceous plants, mangroves) in the order Sapindales. They are characterized by alternate, usually pinnate leaves without stipules, and by syncarpous, apparently bisexual (but actually mostly cryptically unisexual) flowers borne in panicles, cymes, spikes or clusters. Most species are evergreen, but some are deciduous, either in the dry season or in winter. The family includes about 50 genera and 550 species, with a pantropical distribution.

Trichilia is a flowering plant genus in the family Meliaceae. These plants are particularly diverse in sub-Saharan Africa and tropical South America. The genus name "*Trichilia"* is derived from Greek "tricho" referring to the 3-lobed fruits. Several species of *Trichilia* were used in folk medicine and shamanism – e.g. *T. rubescens* against malaria,[1] *T. catigua* was used as aphrodisiac and body stimulant,[2] *T.emetica* as an anticonvulsant[3] and *T.connaroides* as an analgesic.[4]

Trichilia is a genus of trees, widely distributed in south east of Asia. *Trichilia connaroides* (W.&A.) Bentilizen, belonging to family Meliaceae well known as karai, karavilangu found in Kerala, Tamilnadu and Andhra pradesh. *T.connaroides* are distributed in Western ghats and in Central Sahyadris.

Phytochemical screening of leaves have been reported by several authors like isolation of two tetracyclic triterpenoids having a 9,19-cyclopropane ring and two new tetranortriterpenoids-trijugin A and B from chloroform extract of *T.connaroides*.[5] A new limonoid, trichiliton B was isolated from the twigs and leaves of *T. connaroides*.[6] Preliminary phytochemical screening of chloroform extract of *T. connaroides* revealed presence of flavonoid compounds. Flavonoids are known to target prostaglandins, which are involved in the late phase of acute inflammation and pain perception.

Flavonoids are a group of polyphenolic compounds which have the diphenyl propane (C_6-C_3-C_6) skeleton, ubiquitously found in fruits and vegetables. These compounds (aglycones) are commonly glycosylated (at one or more sites with a variety of sugars) and may also be alkoxylated or esterified. As a result over 5000 different flavonoids have been identified in plant materials.[7] The structural difference in each flavonoid family results from the variation in the number and arrangement at the hydroxyl groups and the extent of glycosylation of these groups.[8] Flavonoids are water soluble

polyphenolic molecules containing 15 carbon atoms. Flavonoids belong to the polyphenol family. Flavonoids can be visualized as two benzene rings which are joined together with a short three carbon chain. One of the carbons of the short chain is always connected to a carbon of one of the benzene rings, either directly or through an oxygen bridge, thereby forming a third middle ring, which can be five or six-membered. The flavonoids consist of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids. Red wine contains high levels of flavonoids, mainly quercetin and rutin. The Flavonoids have exhibited antioxidant activity.[9] Flavonoids are becoming very popular because they have many health promoting effects. Epidemiological studies suggest that the consumption of flavonoids is effective in lowering the risk of coronary heart disease.[10-11] Some of the activities attributed to flavonoids include: anti-allergic, anti-tumour, antibacterial, spasmolytic and estrogenic effects.[12-17] They act in plants as photoreceptors, visual attractors, feeding repellants. Many studies have suggested that flavonoids exhibit analgesic, anti-inflammatory, anti-viral, anti-microbial, anti-cancer activities.[18-23] Flavonoids are formed in plants from aromatic amino acids phenylalanine, tyrosine and malonate.

MATERIALS AND METHODS

MATERIALS

Plant:

Leaves of *Trichilia connaroides* (W.&A.) Bentilizen was collected from Kalpeta, Kerala and authenticated from Regional Research Institute, Bangalore. The authenticated leaves were dried in shade and powdered coarsely.

Chemicals and reagents:

All chemicals were procured from Sd fine-chemicals and were of analytical grade. Reagents were freshly prepared and some were purchased from commercial sources.

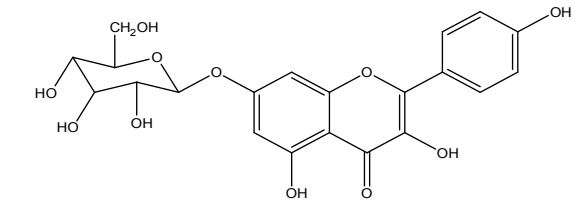
Extraction and Isolation:

The Coarsely powdered leaves (2kg) were first defatted with n-hexane in an aspirator bottle at room temperature (48hr). The coarse powder of leaves was subjected to successive extractions with different solvents like chloroform and methanol. Nearly 80% of the solvent was removed by distillation on a

water bath at atmospheric pressure and the last traces were removed under reduced pressure. The dried residue from methanol extract (30 g) was shaken up with ether, ethyl acetate and n-butanol successively in a separating funnel. Ether and ethyl acetate soluble fractions were separately chromatographed on silica gel columns. Yellow compounds with yellow spots in TLC were obtained. But they were in minor yields. The dried n-butanol extract (15g), was subjected to column chromatography using a column of silica gel. Column was prepared in benzene and eluted with different solvents of increasing polarity, i.e., benzene, benzene: ethyl acetate (1:1), benzene: ethyl acetate (1:4) followed by ethyl acetate. After eluting with different solvents the ethyl acetate eluate on drying gave a yellow solid. The yellow solid was passed through fresh silica gel and finally crystallized from methanol-benzene mixture (1:1), from the crystallized mixture to give populnin (575 mg), m.p 228ºC. **Populnin (FM-1)** NMR 1H C-H 6.62, 1H O-H 5.08, 1H C-H 3.91, 2H CH_2 3.75, 1H C-H 3.47, 1H OH 2.00. Populnin was further subjected to acid hydrolysis and acetylation

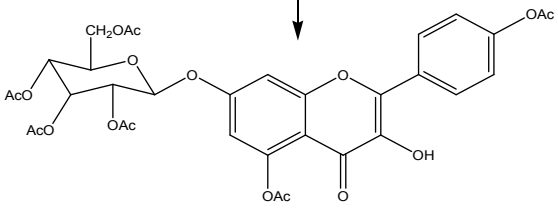
Acetylation:

Populnin (100mg) in dry pyridine (1ml) was treated with acetic anhydride (2ml). Then the mixture was heated on a water bath for 4h. It was kept overnight, few drops of methanol was added and evaporated in vacuo. The dried residue was subjected to column chromatography over silica gel. The chloroform: ethyl acetate eluates (1:1), yielded a solid which was crystallized from chloroform hexane mixture to give the hexa-acetate (MD-1) (60 mg) with a melting point of 172^0C (literature value 173ºC). **Populnin hexa-acetate (MD-1)** Colour yellow, Mol. Formula $C_{33}H_{32}O_{17}$, Mol. Wt. 688, M.P (^{o}C) 173, Mobile phase Chloroform/Ethyl acetate (1:1), R_f Value 0.62. IR O-H (stretching) 3381.57, C-H (stretching) 2922.59, C=O (stretching) 1745.26, C-C (stretching) 1458.29.



POPULNIN

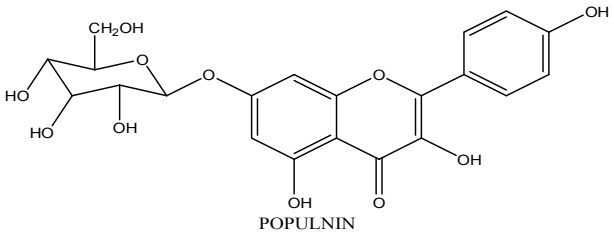
Acetylation



POPULNIN HEXAACETATE

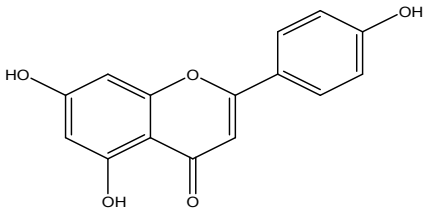
Acid hydrolysis:

Populnin (100mg) in alcohol (5ml) was treated with 1:1 HCl (2ml) and the solution was refluxed on a water bath for 12h. The solution was extracted with ethyl acetate to give a yellow solid which was crystallized from ethanol to yield kaempferol with a melting point of 277°C (literature value 279ºC). The aqueous layer was neutralized and evaporated in vacuo. The sugar was identified as D-glucose by paper chromatography. **5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (MD-2)** Colour yellow, Mol. Formula $C_{15}H_{10}O_6$, Mol. Wt. 286.24, M.P (°C) 280, Mobile phase Benzene/Ethyl acetate (4:1), R_f Value 0.68.



POPULNIN

Acid hydrolysis



KAEMPFEROL

Tests for Flavonoids:

a) **Shinoda test:** To dried extract, 5 ml of 95% ethanol few drops of concentrated HCl and 0.5 g magnesium turnings were added. Pink colour was observed.

b) To small quantity of residue, lead acetate solution was added and was observed for yellow colored precipitate.

c) Addition of increasing amount of sodium hydroxide to the residue was observed as to whether it showed yellow colouration, which was decolorized after addition of acid.

d) Ferric chloride test: To test solution, few drops of ferric chloride solution was added and was observed for intense green colour.

Tests for sugars:

- **Molisch's test:** To 2-3ml extract, few drops of α- naphthol solution in alcohol was added, shaken and concentrated H_2SO_4 was added from sides of the test tube. It was observed for violet ring at the junction of two liquids.
- **Benedict's test:** Equal volume of Benedict's reagent and test solution in test tube were mixed and heated in boiling water bath for 5 min. Solution colour was observed (green, yellow or red) which is dependent on amount of reducing sugar present in test solution.

PHARMACOLOGICAL STUDIES

Animals:

Albino Swiss mice weighing between 18 and 25 g were procured from registered breeders (Venateshwara Enterprises, Bangalore). The animals were housed under standard conditions of temperature (25 ± 2^{o}C) and relative humidity (30-70%) with a 12:12 dark cycle. The animals were fed with standard pellet diet and water. The experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) of KLE University's College of Pharmacy, Bangalore (Reference Number KLEU/COPBLR/17/2010-11) and CPCSEA Registration no. 1712/AC/08/CPCSEA Dated O7.O4.2007.

Acute toxicity studies:

Acute toxicity studies for methanolic extract and populnin, the isolated compound were conducted as per OECD guidelines 423 using albino Swiss mice. Each animal was administered both test compounds dissolved in 50% DMSO. The animals were observed for any changes continuously for 2h and up to 24h for mortality. The methanolic extract and populnin (a flavonol glucoside) did not show any acute toxicity. Common side effects such as mild diarrhoea and depression were not observed.

Grouping of animals:

Albino Swiss mice weighing 18-25 g were divided into six groups of six animals each for both Eddy's hot plate and writhing test. Group-I served as standard, Tramadol (Tramazac®) 5mg/kg body weight[4] was administered by i.p. route in hot plate method and Aspirin 100 mg/kg b.w in writhing test was used as standard[19]. Group-II served as control, Group III and Group IV served for methanolic extract

and populnin. Group III animals received methanolic extract 60mg/kg b.w and Group IV animals received populnin extract 50mg/kg b.w.

Analgesic Activity:

Hot Plate Method:

In thermal method the prescreened albino Swiss mice (reaction time: 3-4 sec) were assigned randomly into six groups of six animals each (n=6). The extract was dissolved in 50% DMSO and was administered intraperitoneally. The delay in reaction time (hind paw licking/jump response) of animals, when placed on hot plate maintained at 55±0.1^{o}C (Eddy's analgesiometer, INCO) was recorded and tabulated. A cut off time was 10 second fixed to avoid damage to the paws. Tramadol 5mg/kg was used as standard analgesic.

% protection against thermal pain = $(T_a - T_b) \times 100/T_b$

Where, T_a – Mean reaction time of test and T_b – Mean reaction time of control

Writhing test using acetic acid in mice:

In acetic acid-induced writhing test, the prescreened albino Swiss mice were assigned into six groups, each containing six animals (n=6). The extract was dissolved in 50% DMSO and was administered subcutaneously. Writhing was be induced 30 min later, by intraperitoneal injection of 0.1 ml of 0.6% acetic acid. The numbers of writhes were counted for 30 min, immediately after acetic acid injection, in all animals. Percentage protection was calculated for all groups and Aspirin 100 mg/kg was used as standard.

Percentage protection= (Writhings in control- Writhings in test/ Writhings in control) × 100

RESULTS AND DISCUSSION

Antinociceptive or analgesic activity of *Trichilia connaroides* was evaluated using both chemical and thermal methods of nociception in mice. These models are used to detect central and peripheral analgesics respectively. Acetic acid induced writhing test is used for detecting both central and peripheral analgesics, where as hot plate model is more sensitive to centrally active analgesics. There

was a significant (P<0.001) analgesic activity with methanolic extract of *Trichilia connaroides* compared to populnin in hot plate method. The populnin analgesic activity was comparable to the standard drug Tramadol (5mg/kg), indicating the involvement of central pain mechanism. It is found that in the experiment Methanolic extract is more active in analgesic activity as compared with the Populnin. Acetic acid induced writhing response in mice indicates that both methanolic extract and populnin produced analgesic activity in dose-dependent manner. The percentage decrease in writhings of both tested compounds was comparable to standard drug aspirin (P<0.001). Methanolic extract 60mg/kg and populnin 50mg/kg protected the animals from acetic acid induced writhings compared to control group. It is found that in the experiment Methanolic extract is more active in analgesic activity as compared with the Populnin. The IR spectrum of populnin showed absorption bands at 3382 cm^{-1} for O-H Stretching (aromatic), 2924.52 for C-H stretching (aliphatic), 1735.62 cm^{-1} for C=O stretching, 1615.09 cm^{-1} C=C stretching, suggesting the presence of hydroxyl and carbonyl groups. In ^{1}H NMR there are well resolved resonance peaks at 6.62 1H (C-H), 5.08 1H (O-H) 3.75 2H (CH_2) 3.91 1H (C-H), 3.47 1H (C-H), 2.00 1H (O-H) δ ppm, which confirm the structure. The isolated compound showed the molecular ion peak at m/z 451.32(M+3) corresponding to molecular formula $C_{21}H_{20}O_{11}$. Acid hydrolysis of populnin gives Kaempferol and D-glucose, which was confirmed by Shinoda test and Molisch test. Acetylation of populnin was identified by IR absorption bands at 3381.57 cm^{-1} for O-H stretching (aromatic), 2922.59 cm^{-1} for C-H Stretching, 1745.26 cm^{-1} for C=0 Stretching, 1458.29 for C-C Stretching. The identification and characterization of populnin was confirmed by IR , LC-MS, NMR.

Table-1: Analgesic effect of *Trichilia connaroides* (W.&A.) Bentilizen and Tramadol in mice by Eddy's hot plate method

Treatment	Dose (mg/kg)	Percentage Increase in reaction time								
		0 min	15 min	30 min	45 min	60 min	120 min	180 min	240 min	360 min
Standard (Tramadol)	5	3.32±0.81	5.66±1.21	5.83±1.16	6.16±0.75	6.10±0.63	5.83±03	9.2±0.84	11.0±1.09**	6.6± 1.03**
Control	50% DMSO	3.16±0.98	3.33±0.81	3.33±0.51	3.33±0.51	3.45±0.81	3.48±0.81	3.44±0.75	3.66± 0.63	3.83±0.62
Methanolic Extract	60	2.83±0.75	3.66±0.51	5.16±0.75	6.33±0.81	7.83±0.75	9.66±0.81	12.3±1.63	12.17±1.7**	9.16±2.43 **
Populnin	50	3.16±0.98	5.33±0.81	5.32±0.51	4.66±0.51	5.16±0.81	4.54±0.81	4.33±0.75	4.23± 0.63*	4.12±0.62*

n=6. Values represent Mean± SD in each group (P<0.005) when compared to control and (P<0.001) when compared to standard.

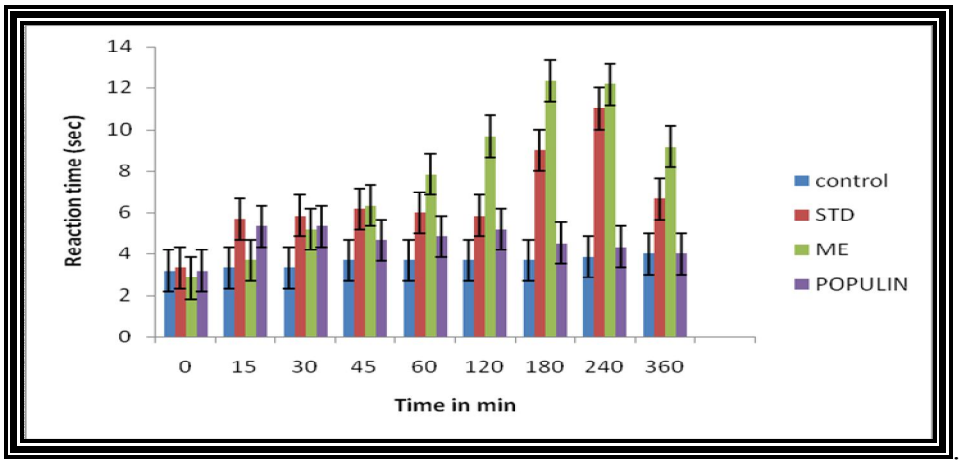


Figure-2: Effect of *Trichilia connaroides* (W.&A.) Bentilizen in mice by Eddy's hot plate method

Figure represents Mean ± SEM in each group (P<0.005) when compared to control and (P<0.001) when compared to standard

Table 2: Analgesic effect of *Trichilia connaroides* (W.&A.) Bentilizen and Aspirin in mice by Acetic acid induced writhing test

Treatment	Dose (mg/kg)	Number of Writhes	Percentage protection
Standard(Aspirin)	100	10.50 ± 0.82**	68.61 ± 1.82**
Control	50% DMSO	32.50 ± 1.71	-
Methanolic Extract	60	14.60 ± 0.63*	34.45 ± 0.46*
Populnin	50	14.27 ± 0.38	32.28 ± 0.28

n=6. Values represent Mean± SD in each group (P<0.005) when compared to control and (P<0.001) when compared to standard.

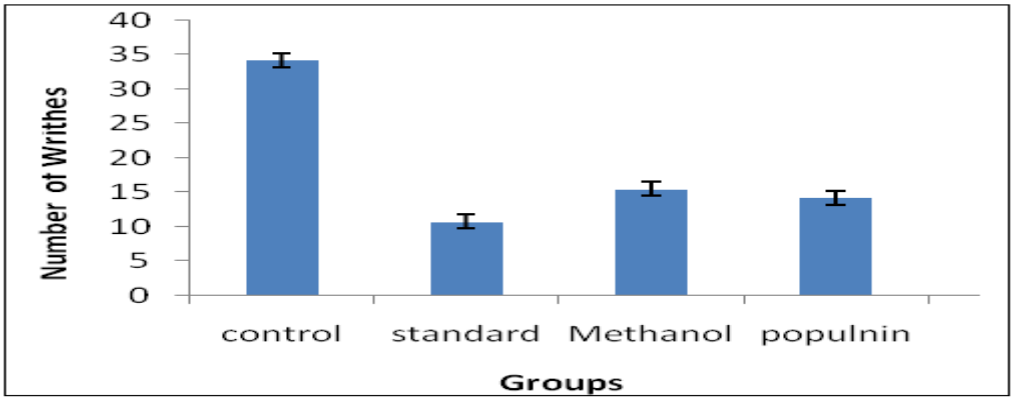


Figure-3 **Effect of *Trichilia connaroides* (W.&A.) Bentilizen in mice by Acetic acid induced writhing test**

Figure represent Mean ± SEM in each group (P<0.005) when compared to control and (P<0.001) when compared to standard.

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