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PHYTOCHEMICAL EVALUATION, ANTIINFLAMATORY ACTIVITY, AND DETERMINATION OF BIOACTIVE COMPONENTS FROM LEAVES OF MUSSAENDA FRONDOSA

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Abstract:

The present study is focused on phytochemical screening of aqueous and methanolic leaf extracts of Mussaenda frondosa (L) and anti-inflammatory activity of different leaves extracts of Mussaenda frondosa, by using carageenin indused paw edema method. The preliminary phytochemical investigation showed the presence of steroids and phenolic compounds, glycosides, carbohydrates etc.The compounds β-sitosterol, lupeol, mussendoside, mussendoside-M, sanshide-methyl ester, 3-palmitoyl lupeol have been isolated from different leaf extracts of Mussaenda frondosa(L). Further, these isolated structures were established by spectral analysis and direct comparison with authentic samples. This is the first report of occurrence of these compounds from Mussaenda frondosa(L).From the anti-inflammatory activity method i.e.carageenin indused paw edema method, the petroleum ether, ethyl acetate extracts were exhibited significance anti-inflammatory activity ($P < 0.01$). Due to presence of steroids and flavonoidal glycosides. Whereas butanol extract showed marked anti-inflammatory activity ($P < 0.05$) when compared standard. The results clearly indicate that ethyl acetate and petroleum ether extracts of Mussaenda frondosa are effective against inflammatory diseases.

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INTRODUCTION:

Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The plants are indispensable to man for his life. Nature has provided a complete store-house of remedies to cure all ailments of mankind. The knowledge of drugs has accumulated over thousands of years as a result of man's inquisitive nature so that today we possess many effective means of ensuring health care [1]. Nature always has been a valuable source of drugs and despite the unprecedented opportunities afforded by medicinal chemistry continues to deliver lead compounds Historically, ethnobotanical leads have resulted in three different types of drug discovery Mussaenda is a genus of flowering plants in the Rubiaceae family. They are native to the African and Asian tropics and subtropics. Several species are cultivated as ornamental plants. It is a smaller shrub 1.5 to 2 metres tall by 1.5 to 2 metres wide. Like all other Native to India, this shrub grows to a height and spread of 6 ft (1.8 m). In summer it bears clusters of orange-yellow tubular flowers beside large, white bracts, set among pale green, oval leaves; berries follow the bloom Mussaenda species, petals are visible as pale white colored leaves just below the flower. Mussaenda frondosa (L) is a species of flowering plant in the Rubiaceae family.It is common flowering shrub native to India, Srilanka, Nepal, Cambodia, Malaysia and Indonesia.It has become one of the most popular flowering shrubs in Florida gardens and landscapes.

Medicinal properties: The whole plant of wild Mussaenda is used for cough, bronchitis, fever, wounds, ulcers, leucoderma, pruritis, jaundice and anti-inflammatory activity. Leaves make excellent herbal shampoo it is used in traditional medicine as anti-diarrhoeal, diuretic, and in lithiasis. It is valued for cough, as a vermifuge for children, in the treatment of headache, and in arsenic poisoning. The herb is also used in malaria and skin diseases and snake bites [2].

Inflammation is considered as primary physiologic defense mechanism that helps body to protect itself against infection , burn, toxic chemicals , allergens are others noxious stimuli.There are various compounds to an inflammatory reaction that can contribute to the associated symptoms and tissue injury.

Acute toxicity studies:

The preliminary pharmacological studies were conducted to assess the acute pharmacological effects and LD_{50} of the isolated compounds of (petroleum ether, ethyl acetate extract of ***mussaenda frondosa***(L) leaves).The acute toxicity study was carried out in adults female albino rats by up and down methods(OECD guidelines 425) [3]. The animals were fasted overnight and next day isolated compounds of ***mussaenda frondosa*** (L) (suspended in 0.6%w/v sodium CMC) were administered orally at different dose level. Then the animals were observed continuously for three hours for general behavioral, neurological, autonomic profiles and then every 30 mins for next three hours and finally death after 24 hour.

Carageenin -indused rat paw edema method (acute inflammatory model) [10-12].

Edema formation, leukocyte infection and granuloma formation represent such compounds of inflammation. Edema formation in the paw is the result of a synergism between various inflammatory mediators that increase vascular permeability and / or the mediators that increase blood flow. Several experimental models of paw edema have been described. Carageenin- induced paw edema is widely used for determining the acute phase of inflammation.[4]

MATERIALS AND METHODS:

Collection and identification of plant material:

The whole plant, ***Mussaenda frondosa Linn*** was collected from land scape of local areas of Karnataka and authenticated Voucher specimens are kept at the Malla Reddy College of Pharmacy, Dhulapally, Hyderabad, A.P., India.

Extract Preparation:

The shade dried leaves of *Mussaenda* were reduced to fine powder and around 5.0 kg were subjected to continuous successive extractions with different solvents of increasing polarity like Petroleum ether, ethylacetate and butanol into 15 batches of each 250-300 gm each in a soxhlet extractor.These extracts were filtered and concentrated to dryness in rotavapour .These dried extracts were stored in desiccators

Phytochemical Analysis

Qualitative Analysis:

All the above different extracts were subjected to preliminary qualitative chemical investigation.

Test for Sterols [5].

a. Salkowaski test:

2 mg of dry extract of leaves was shaken with chloroform. To the chloroform layer sulphuric acid was added slowly by the sides of the test tube. Formation of red color indicates the presence of sterols.

b. Liberman-Burchard's test [6]:
2 mg of dry extract of leaves was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green color indicates the presence of steroids.

Test for Flavonoids:

a. Shinodas's test:
In a test tube containing 0.5 ml of the extract of leaves, 10 drops of dilute hydrochloride acid was added, followed by a piece of magnesium. Formation of pink or reddish or brown color indicates the presence of flavonoids[7]

Test for Tripernoids:
a. Liberman-Burchard's test [7]:
2 mg of dry extract of leaves was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of violet colored ring indicates the presence of triterpenoids.

Test for Saponins:

a. Foam test:
In a test tube containing about 5 ml of extract of leaves, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like foam/froth indicates the presence the presence of saponins.

Test for Carbohydrates [8]:

a. Molish's test:
A test tube containing 2 ml of the extract of leaves, 2 drops of freshly prepared 20 % alcoholic solution of alpha–naphthol was added. 2 ml of conc. Sulphuric acid was added, so as to form a layer below the mixture. Red-violet ring appeared indicating the presence of carbohydrates, which disappeared on the addition of excess of alkali.

b. Benedict's test:
To 2 ml of the extract of leaves, 5 ml of Benedict's solution was added and boiled for 5 minutes. Formation of brick red colored precipitate indicates the presence of carbohydrates.

c. Fehling's test:
To 2 ml of extract of leaves, 1 ml of mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes. Formation of red or brick red colored precipitate indicates the presence of reducing sugar.

Tests for Alkaloids [9,10]:
a. Mayer's test:
To a few drops of the Mayer's reagent, 2 mg of extract of leaves was added. Formation of white or pale yellow or buff colored precipitate indicates the presence of alkaloids.

b. Wagner's test:
2 mg of extract of leaves was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent were added. A yellow or brown colored precipitate indicates the presence of the alkaloids.

c. Hager's test:
To 2 mg of the extract of leaves taken in a test tube, a few drops of Hager's reagent were added. Formation of yellow colored precipitate confirms the presence of alkaloids.

d. Dragendroff's test:
To 2 mg of extract of leaves, 5 ml of distilled water was added; 2 M Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendroff's reagent was added. Formation of orange or orange red colored precipitate indicates the presence of alkaloids.

For Tannins [11]:
To 1-2 ml of the extract of leaves, few drops of 5 % w/v FeCl3 solution was added. A green colored indicates the presence of gall tannins, while brown color indicates the presence of pseudo tannins.

Test for Proteins:

a.Millon's test:
1.ml of ethanolic extract of leaves was dissolved in 1 ml of distilled water and 5-6 drops of Millon's reagent were added. Formation of white colored precipitate, which turns red on heating, indicates the presence of proteins.

b.Biuret test:
To 1 ml of hot extract of leaves, 5-8 drops of 10 % w/v sodium hydroxide solution was added, followed by 1 or 2 drops 3 % w/v copper sulphate solution were added. Formation of a violet red color indicates the presence of proteins.

Test for Resins:
1 ml of extract of leaves was dissolved in acetone and solution was poured in distilled water. Turbidity indicates the presence of resins.

Test for Starch:
0.01 g of iodine and 0.075 g of potassium iodide were dissolved in 5 ml of distilled water and 2-3 ml

of extract of leaves was added. Formation of blue color indicates the presence of starch.

Test for Glycosides [12]:

a. Legal test.

Leaves extract is dissolved in pyridine; sodium nitroprusside solution is added to it and made alkaline. Pink red color is produced.

b. Baljet test:

To the leaves extract, sodium picrate solution is added; yellow to orange color is produced.

c. Borntrager test [13]:

Added a few ml of dilute sulphuric acid to the test solution, Boil, filter and extracted the filtrate with ether or chloroform. The organic layer is separated to which ammonia is added; pink, red or violet color is produced in organic layer.

d. Keller Killani test [14]:

Sample was dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of liquid reddish brown colour was produced which gradually becomes blue.

The preliminary qualitative chemical investigation of petroleum ether, ethyl acetate and butanolic extracts of ***Mussaenda frondosa Linn***, was showed in Table.1

Table no.1: Preliminary qualitative chemical investigation of different solvent extracts of *Mussaenda frondosa Linn* leaves

S.No	Tests	Petroleum ether	Ethyl acetate	Butanol
1	**Test for Alkaloids**	-	-	-
a	Mayers test	-	-	-
b	Wagners test	-	-	-
c	Hagers test	-	-	-
d	Dragen draffs test	-	-	-
2	**Test for Glycosides**	+	+	+
3	**Test for Sterols**	+	-	-
a	Salkowaski test	+	+	-
b	Libermann burchard test	+	+	+
c	Sulphur test	-	-	-
4	**Test for Amino acids**	-	-	-
5	**Proteins**	-	-	-
a	Millons test	-	-	-
b	Biuret test	-	-	-
6	**Test for Tannins**	-	-	-
7	**Test for Flavanoids**	+	+	-
a	Shinoda test	+	+	+
8	**Test for Carbohydrates**	+	+	+
a	Molish test	+	+	+
b	Fehlings test	+	+	+
C	Benedicts test	+	+	+
9	**Test for Triterpenoids**	-	+	+
a	Libermann buchard test	+	+	+
10	**Test for Starch**	+	+	+

NOTE: (+) indicates present (-) indicates absent

Table no.2: The results of qualitative chemical investigation of Petroleum ether, ethyl acetate and butanolic extracts of *Mussaenda frondosa Linn* leaves.

Petroleum ether extract	Glycosides, sterols, carbohydrates, flavanoids
Ethyl acetate extract	Glycosides, carbohydrates, flavanoids triterpinoids
Butanol extract	Glycosides, carbohydrates, flavanoids Triterpenoids, alkaloids

Anti-Inflammatory activity:

Carageenin- Induced Paw edema method.

Wistar rats of either sex weighing between 100-150g are used. The animals are starved overnight with water being provided *ad libitum*.The test compounds and standard drugs are administered by oral or intra peritoneal route. Thirty min later the rats are challenged by 0.05ml of 1% solution of carageenin on the plantar surface of the left hind paw. The paw was marked with ink at the level of lateral malleolus and immursed in the mercury column of plethysmometer for measuring the paw volume. The paw volume was measured immediately after the carageenin injection and then at 2, 3, 4 and 6 h. the peak effect of carageenin usually occurs of 3h after the injection.The increase in paw paw volume at 3h was calculated as percentage compared with the volume measured immediately after the injection of carageenin for each animal and control group was calculated for each dose of the drug. A dose response curve was plotted and used for the determination of ED50 values [15-22].

Table 3: detailed explanation of animal segregation

S.NO	GROUP	TREATMENT
1	GROUP-I	Served as control receiving 0.2%w/v of carageenin
2	GROUP-II	Served as positive control and received Diclofenac standard drug(100mg/kg) orally, as suspension in acacia (0.5ml 0.1%v/v)
3	GROUP-III	Animals were treated with (200mg/kg b.w) of 0.5ml of (β-sitosterol) 0.1% w/v solution , orally)
4	GROUP-IV	Animals were treated with (200mg/kg body weight) of (lupeol) 0.1% w/v solution , orally)
5	GROUP-V	Animals were treated with (200mg/kg body weight) of (mussendoside) 0.1% w/v solution , orally)
6	GROUP-VI	Animals were treated with (200mg/kg body weight) of (mussendoside-M) 0.1% w/v solution , orally)
7	GROUP-VII	Animals were treated with (200mg/kg body weight) of (3-palmitoyl lupeol) 0.1% w/v solution , orally)

RESULTS AND DISCUSSION:

In the acute inflammation model i.e carageenin-indused hind rat paw edema test, statistically. It was found that,(β- **sitosterol, lupeol, mussendoside, mussendoside-M**) of *mussenda frondosa* (L) leaves at dose of 200mg/kg exhibited significant (P<0.01) reduction in paw volume which is comparable to diclofenac (100mg). The compounds (**samshide-methyl ester, 3-palmitoyl lupeol**) of mussenda frondosa (L) leaves at dose of 200mg/kg exhibited a marked (P<0.05), inhibition of paw volume which was comparable to diclofenac sodium (100mg). Hence, the anti-inflammatory effect of isolated compounds **(β- sitosterol, lupeol**, **mussendoside, mussendoside-M, 3-palmitoyl lupeol, sanshide methyl ester)** of *Mussenda frondosa* (L) leaves seems to support the use of inflammatory ailments.

Table no 4:Anti-inflammatory activity of isolated compounds of *Mussaenda frondosa* leaves in carageenin induced paw edema method.(% inhibition of paw volume)

GROUPS	DOSE (MG/KG)	0 MIN	15 MIN	30 MIN	60 MIN	2 H	4 H
Control	000	0.039± 0.0001	0.041± 0.0003	0.043± 0.0001	0.047± 0.0001	0.050± 0.0004	0.052± 0.0007
Standard	100	0.003± 0.001*	0.023 ±0.0002*	0.030± 0.0001**	0.023± 0.0001**	0.021± 0.0008**	0.017± 0.0003**
ER-1	200	0.036± 0.0001*	0.033± 0.0004*	0.030± 0.0004*	0.027± 0.0009**	0.027± 0.0009**	0.025± 0.0005**
ER-2	200	0.035± 0.0001*	0.030± 0.0001**	0.025± 0.0001**	0.021± 0.0001*	0.021± 0.0001**	0.019± 0.0004**
ER-3	200	0.037± 0.0001*	0.026 ±0.0001**	0.026± 0.0001**	0.022± 0.0001**	0.022± 0.0001**	0.020± 0.0003**
ER-4	200	0.021± 0.0004*	0.027± 0.0003**	0.024± 0.0007**	0.017± 0.0003**	0.017 ±0.0003**	0.015± 0.0003**
ER-5	200	0.037± 0.0003*	0.029± 0.0003*	0.028± 0.0003*	0.025± 0.0003**	0.025± 0.0003**	0.023± 0.0003**

Statistical analysis was done by ANOVA followed by Dunnet's test. All the values are expressed as mean ± SEM.*P<0.05,**P<0.01. When compared to control.

Extraction and preliminary Qualitative analysis:
The shade dried leaves of ***Mussaenda frondosa*** *(*L*)* was subjected to successive continuous hot extraction by using different solvents like petroleum ether, ethyl acetate, butanol in soxhlet apparatus. All these extracts were concentrated and calculated for their percentage yield. The yields were found to be 50g, 42g, and 40g respectively.
The results of phyto-chemical investigation of petroleum ether.ethyl acetate, butanol extracts shows the presence of steroids, glycosides, flavonoids, phenolic compounds etc.,

Phyto-chemical characterization of different extracts and isolated compounds of *Mussaenda frondosa* (L) leaves:
From petroleum ether extract: Three isolated compounds were obtained.
From fraction A:
A sticky mass compound was resulted as single compound from the fraction A which was further recrystalised from acetone, M.P: 271-272ºC.
IR spectra shows bands at 2983.52 and 2934.54 cm^{-1} indicates C-H str. in CH_2 and CH_3, 1447.42 cm^{-1} indicates C-H deformation in CH_3, 1372.26 cm^{-1} indicates C-C stretching, 1232.21 cm^{-1} indicates C-O stretching in acetate.
^{1}HNMR spectral data, one vinylic proton of appears as multiplet at 5.12 δ ppm, ,H-6),3.68(1H,s,OH, H-3),3.25(1H,m,CH,H-3),2.03 to 1.01 (30 H,m,CH2&CH protons),0.99 to 0.76(18H,m,6xCH3 group).
^{13}CNMR spectral data exhibited the presence of 29 carbon signals of both aliphatic and cyclic carbons moiety carbons are in their respective ppm. ^{13}C NMR spectral data matched with exactly with that of **β-sitosterol**.

Further the structure was confirmed as **"β-sitosterol"** with its molecular ion peak 414 [M^+] (60%) and molecular formula $C_{29}H_{50}O$.

From fraction B: Whereas light yellowish sticky mass steroidal compound which was further recrystalized with acetone, M.P. 240-270ºC.
^{1}HNMR spectral data shows peaks 5.31(d,1H,C=CH2 group, C-29,1a),4.31(m,1H,C=CH2 group,C-29,1b),3.65(m,1H,OHgroup),3.25(s,1H,H-3),2.40(s,1H,H-29) , 2.1(m,3H,CH3&CH=CH2,H-30),1.40 to 1.0(m,12H,4xCH3 group),2.6 to 1.0(m,CH&CH2 protons),0.99 to 0.88(m,6H,2xCH3 groups,H-23,24).
^{13}CNMR spectral data exhibited the presence of 30 carbon signals of both aliphatic and cyclic carbons are in their respective ppm. ^{13}C NMR spectral data matched with exactly with that of **Lupeol**.

Further the structure was confirmed as **"Lupeol"** with its molecular ion peak 426 [M^+] (55%), and molecular formula $C_{30}H_{50}O$

From fraction C: A light green colour compound obtained from **fraction C** after recrystallisation with acetone, M. P 238 - 240 °C.
^{1}HNMR spectral data, 3.76(s, 1H,OH group at H-26,31),3.65(s,1H,OH group at H-3),3.56(m,CH2 protons, H-20), 3.30 to 3.18(m,2H, H-26,3),2.27 to 1.18(m,CH2 & CH protons of cyclic ring), 1.13(m,6H,2xCH3 group,H-27,28),0.99(m,6H,2xCH3 group,H-29,30),0.95(m,3H,CH3 group, H-22).
^{13}CNMR spectral data exhibited the presence of 30 carbon signals of aliphatic and cyclic carbons in their respective ppm. Further the structure was confirmed as **"Mussendoside".**

From ethyl acetate extract: Two isolated compounds were obtained.

From fraction A: light yellow color sticky compound was resulted from fraction A and its M.P. 273-274ºC.
In IR 3442 cm^{-1} (OH stretching) ,3364 cm^{-1} (NH stretching), 1740cm^{-1} (cyclo pentanone)
^{1}HNMR spectral data, one proton of NH group appears as singlet at 8.2 δ ppm,two protons of CH groups appears as multiplet at 6.2 to 6.0 δ ppm,two protons of lactone ring appears as multiplet at 4.7 to 4.2, one proton peak of hydroxyl group appears as singlet at3.7 δ ppm.
^{13}CNMR spectral data exhibited the presence of 35 carbon signals of cyclic carbons in their respective ppm. Further the structure was confirmed as **"Mussaenoside- M"** with its molecular ion peak at 537 [M^+] (10%), which corresponds the molecular formula ($C_{35}H_{55}NO_3$), and confirmed with authentic sample of **Mussaenoside-M**.

From fraction B:
A brown color amorphous compound obtained from **fraction B**. M. P 213 - 215 °C. IR shows absorption at 2879 cm^{-1}, denotes C-H streching, at 1736 cm^{-1} denotes C-O-O stretching ester, at 1653 cm^{-1} indicates C=C stretching.
^{1}HNMR spectral data, Two =CH_2 protons appears as singlet at 5.2 & 4.7 δ ppm, protons of CH_2 &CH groups appears as multiplet at 2.1 to 1.2 δ ppm, one proton peak appears as multiplet at 3.9 to 3.6 δ ppm.
^{13}CNMR spectral data exhibited the presence of 46 carbon signals of aliphatic and cyclic carbons in their respective ppm. Further the structure was confirmed as **"3-palmitoyl lupeol"** with its molecular ion peak at 665 [M^+] (2%), which corresponds the molecular

formula ($C_{46}H_{80}O_2$), and confirmed with authentic sample of **3-palmitoyl lupeol**.

From butanolic extract: One compound was obtained.

One fraction is eluted which reddish brown is colored iridoid glycoside compound.

IR shown bands absorption at 3411 cm^{-1} indicates O-H stretching, at 1711 cm^{-1} C=0 stretching, at 134 cm^{-1} indicate C-O stretching.

^{1}HNMR spectral data, one proton appears as doublet at 7.52 ppm, two protons of CH_2 appears as multiplet in between 4.29 to 3.82 ppm, one proton of OH group at 3.45Ppm.

^{13}CNMR spectral data exhibited the presence of 18carbon signals of both aliphatic and cyclic carbons are in their respective ppm. ^{13}C NMR spectral data matched with exactly with that of Shanshiside methyl ester.

Further the structure was confirmed as **"Shanshiside methyl ester"** with its molecular ion peak 406 [M^+] (98%) and confirmed with authentic sample of shanshiside methyl ester.

Anti–inflammatory activity:

Different extracts were screened for **anti-**inflammatory activity by using carageenin induced paw edema method

Carageenin induced paw edema method.

The ethyl acetate and petroleum ether extracts showed significant anti-inflammatory activity ($P < 0.01$). Whereas butanol extract exhibited marked activity ($P < 0.05$)

CONCLUSION:

The shade dried leaves of ***Mussaenda frondosa*** was subjected to successive continuous hot extraction by using different solvents like petroleum ether, ethyl acetate, butanol. The chemical investigation of leaves of ***Mussenda frondosa*** lead to the isolation of six compounds **β-sitosterol, lupeol, mussaendoside, mussaendoside-M,3-palmitoyl lupeol, sanshide-methyl ester** . The constituents isolated and characterized from the leaves of the plant ***Mussaenda frondosa*** can be categorized under triterpenoids, flavonoids, steroids and phenolic compounds.

Mussaendoside, lupeol, β-sitosterol were isolated from petroleum ether extract by column chromatogram using petroleum ether: acetone as mobile phase.**Mussendoside-M, 3-palmitoyl lupeol** were isolated from ethyl acetate extract by column chromatography using ethyl acetate::chloroform as mobile phase. **Samshide-methyl ester** were isolated from ethanol extract by column chromatography using methanol : ethyl acetate as mobile phase.The different extracts of leaves of ***Mussaenda frondosa*** were subjected to *in-vitro* anti-inflammatory activity i.e. carageenin induced paw edema methods. Petroleum ether and ethyl acetate extracts shows significance response ($P<0.01$) of anti – inflammatory activity .Butanol extract showed marked anti inflammatory activity($P<0.05$).These extracts synthesis of 5-HT and bradykinin synthesis & histamine release can reduced. The compounds showed good anti-inflammatory effect on wistar rats. It will helps to treat inflammatory reactions like prurities, injury, swelling and local effects.Hence the compounds lupeol, Mussendoside-M, β-sitosterol, mussaendoside, 3-palmitoyl lupeol,sanshide methyl ester possesses anti-inflammatory activity by reducing the secretion of pro inflammatory cytokines and α-TNF24,25 which may responsible for inflammation.

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