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Phytochemical, Cytotoxic, anti- HSV-1 (Herpes Simplex Virus type- 1) and anti bacterial studies of *Terminalia laxiflora Engl. and Diels.*

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ABSTRACT

Plan: *The aim of the present research was focused on cytotoxic, antiviral and antibacterial properties and the main phytoconstituents of Terminalia laxiflora leaf methanolic extract.*
Methodology: *The methanolic 80% extract of Terminalia laxiflora leaves was tested for cytotoxicity evaluation in Vero cells in-vitro, anti-HSV-1 activity and antibacterial activity determinations against some bacteria strains and also phytoconstituents were detected.*
Outcome: *The results showed that Terminalia laxiflora leaves methanol 80% extract showed CC_{50}= 1851 μg/mL and also inhibited completely the development of the HSV-1 induced cytopathic effect at concentration of 238 μg/mL while the extract had no effect on bacterial strains. Phytochemical analysis of the extract revealed the presence of carbohydrates, tannins, flavonoids, alkaloids, triterpenes and chromatographic separation of the extract of Terminalia laxiflora leaves resulted in the isolation and identification of β-sitosterol, m-gallate, gallic acid, ellagic acid and five flavonoids, quercetin, vitexin, iso vitexin, quercetin 3-O-α-rhamnoside and rutin. This research may be useful in the development of new and effective antiviral agents.*
Keywords: *Terminalia laxiflora, leaves, cytotoxicity, anti-HSV-1, antibacterial, phytoconstituents, methanolic extract*

1. INTRODUCTION

Viral diseases are now considered to be one of the most important and dangerous problems worldwide due to the difficulty of controlling most of them. Because viruses are obligate intracellular parasites, antiviral agents must be capable of selectively inhibiting viral function without damaging the host. Herpes simplex virus type 1 (HSV-1) is widespread, enveloped and double stranded DNA agents which cause various infections in human. The virus causes recurrent infections of the nervous system located around the lips, in the eyes, in the mucous membrane of the oral cavity and genital as well[1].

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The drugs found to be clinically useful in the treatment of HSV-1 are synthetic nucleosides such as acyclovir (ACV). The sever side effects and the emergence of drug-resistance mutants during long-term medication with these drugs have often limited their administration to patients[2,3]. Thus, the development of novel antiviral agents against this virus is still an important area of research.

One of the possible methods which can be used for the discovery of active substances is the screening of plant extracts for antiviral activity followed by bioassay guided fractionation of active extracts to identify the active substance. In searching for natural products as potential antiviral agents, *Terminalia* is a genus of large trees of the flowering plant family Combretaceae, comprising around 100 species distributed in tropical regions of the world. *Terminalia laxiflora* is common indigenous tree from Combretaceae family and it is native to West Africa. In folk medicine, *Terminalia laxiflora* leaves are used against dysentery[4,5]. The aqueous extract of *Terminalia laxiflora* stem bark has shown antibiotic action against *Sarcina lutea* and *Staphylococcus aureus* and various parts of the plant are used for tubercular coughing with vomiting and bloody sputum[4,5].

Few reports about chemical constituents and biological activities of the plant, root bark extract of the plant showed antimicrobial activity[6]. The aim of the present study is to determine cytotoxicity, anti-Herpes simplex virus type 1 (HSV-1) and antibacterial activities of *Terminalia laxiflora* leaves methanol 80% extract and also to investigate the phytoconstituents present in the *T. laxiflora* leaves.

2. MATERIALS AND METHODS

2.1. General experimental procedures

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). Spectroscopic data: NMR–Varian, 400 MHz. MS (Finnigan MAT SSQ 7000, 70ev). Silica gel (60-200 mesh, Merck), Sephadex LH-20 (Sigma), Thin Layer Chromatography (TLC): pre-coated sheets of silica gel 60 F_{254} (Merck), Paper Chromatography whatmann No. 1.

2.2. Plant material

T. laxiflora leaves were collected from Al-Zohiriya garden, Giza, Egypt in May 2011. The plant was identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereeza Labib Consultant of Plant Taxonomy at the Ministry of Agriculture and Director of Orman botanical garden, Giza, Egypt. A voucher specimen was deposited in the herbarium of Al-Zohiriya garden, Giza, Egypt.

2.3. Preparation of T. laxiflora leaves methanol 80% extract

Air dried powder of *T. laxiflora* leaves (950 g) was extracted with methanol: water 80:20 (v/v) at room temperature several times until exhaustion by maceration. The extract was concentrated under reduced pressure to give 54 g of crude extract and phytochemical screening of the extract was done according to methods described by Ayoola[7].

2.4. Phytochemical Characterization of T. laxiflora leaves methanol 80% extract

The crude methanol 80% extract (54 g) was defatted with n-hexane several times and the defatted extract (42 g) was subjected to silica gel column chromatography eluting with different amounts of dichloromethane, ethyl acetate and methanol gradually. The fractions that showed similar thin layer chromatography (TLC) were collected and according to that four fractions were obtained. Fraction 1 (1.2 g) eluted with dichloromethane: ethyl acetate (80:20 v/v) gave compound 1 (β-sitosterol, 21 mg) and further elution with dichloromethane: ethyl acetate (60:40 v/v) gave compound 2 (m-gallate, 28 mg). Fraction 2 (1.32 g) eluted with ethyl acetate : dichloromethane (50:50 v/v) gave compound 3 (gallic acid, 23 mg) and further elution with ethyl acetate : dichloromethane (70:30 v/v) gave compound 4 (ellagic acid, 27 mg). Fraction 3 (1.75 mg) eluted with ethyl acetate gave compound 5 (quercetin, 18 mg) and further elution with ethyl acetate:methanol (90:10 v/v) gave compound 6 (apigenin 8 C-β-glucoside (vitexin), 20 mg) and further elution with ethyl acetate : methanol (85:15 v/v) gave compound 7 (apigenin 6 C-β-glucoside (isovitexin), 22 mg). compound 8 (quercetin 3-O-α-rhamnoside, 25 mg) and compound 9 (rutin, 40 mg) was isolated from fraction 4 (1.42 g) by elution with ethyl acetate : methanol (75:25 v/v) and elution with ethyl acetate : methanol (50:50 v/v). All the isolated compounds were purified on sephadex LH–20 column using methanol and different mixtures of methanol and distilled water.

2.5. General method for acid hydrolysis of flavonoid glycosides

5 mg of each compound 6, 7, 8 and 9 in 5 ml 10% HCl was heated for 5h. The aglycones were extracted with EtOAc and identified by co-TLC with authentic standards. The sugars in the aqueous layer was identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (*n*-BuOH-AcOH-H_2O 4:1:5 upper layer).

2.6. Biological assays

Cytotoxicity assay: Vero cells were cultured in 96-well microplates, and the monolayers were incubated for 72 h at 37^{o}C and 5% CO_2 with DMEM containing 5% fetal bovine serum (FBS), penicillin G (100 IU/ml), enrofloxacin (10 μg/ml) and amphotericin B (1.25 μg/ml) with 2-fold serial dilutions of the compounds at different concentrations, ranging from from 3.8 to 1900 μg/ml to methanol 80% of *T. laxiflora* leaf extract. For this, 3 repetitions of 8 wells were used for the evaluation of extract dilution. The *in vitro* toxicity of methanol 80% of *T. laxiflora* leaf extract which was sterilized by filtration through PVDF membranes (pore size 0.22 μm), was determined by quantifying the viable cells using 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is converted into a purple formazan by mitochondrial dehydrogenases[8]. Fifty percent cytotoxic concentration (CC_{50}) was defined as the extract concentration which could reduce by 50% the number of viable cells, when compared with a control without it, and it was calculated by regression analysis of the dose-response curves.

2.6.1. Antiviral assay:

The preliminary screening of the antiviral activity of *T. laxiflora* methanol 80% leaves extract against HSV-1 was carried out on confluent monolayers of Vero cells in 96-well microplates by adding the highest non-toxic concentration determined by the MTT method (238 μg/mL) during the HSV-1 ($100xTCID_{50}$) adsorption step and after adsorption in the same concentrations within the maintenance medium (DMEM with 5% FBS added by antibiotics and antifungal). The viral replication was performed for 72h at 37°C and 5% CO_2 incubation. The inhibition of the HSV-1 replication was related to the absence of the viral induced cytopathic effect, evaluated by observation of the monolayers under microscope. After that, dilutions of this extract with lower concentrations (207 to 13 μg/mL) were tested against HSV-1, using the same experimental conditions described above. Fifty percent effective concentration (EC_{50}) was defined as the extract concentration which could reduce by 50% the number of infected wells (been considered infected those showing any virus-induced cytopathic effect), when compared with a control without it, and it was calculated by the endpoint titration method[9]. Acyclovir was titrated as a control.

2.6.2. Antibacterial assay:

The antibacterial activity of methanol 80% of *T. laxiflora* leaf extract was evaluated against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) strains by the disk-diffusion method. Briefly, sterilized filter paper disks of 6 mm diameter were impregnated with 10 μL of the control substances (water, 0,005% enrofloxacine or 20% clorexidine) or with extract solutions (580 and 238 μg/mL) previously sterilized by filtration on PVDF membranes with pore diameter of 0.22 μm. The impregnated disks (n=3) were positioned on the top of the Petri dishes containing 25 mL of Mueller-Hinton Agar previously seeded with a bacterial suspension adjusted to the 0.5 degree of the McFarland turbidity scale, which corresponds to a concentration of $1.5x10^8$ CFU/mL. The plates were incubated at 35°C for 24 h and after that, the diameter of the zone of inhibition of the bacterial growth around the discs were determined.

2.7. Statistical analysis

All biological experiments were statistically expressed as mean ± standard deviation, and analyzed by Student´s *t* test with $P<0.01$. Variables exceeding the upper quantification limit were considered statistically significant.

3. RESULTS AND DISCUSSION

The present investigation evaluated the cytotoxic, antiviral and antibacterial properties of *Terminalia laxiflora* leaves methanol 80% extract *in vitro* and also detected the main bioactive phytoconstituents from *T. laxiflora* methanol 80% leaves extract.

3.1. Cytotoxicity studies

T. laxiflora methanol 80% leaves extract showed no statistically significant difference in relation to the control of cellular viability without extract up to 238 µg/mL (Fig. 1), although an alteration of the normal morphology of the cells related to a granulation of the cytoplasm was observed even at this non-toxic concentration, presenting CC_{50} = 1851µg/mL. This value is above that reported for the aqueous extract from the fruits of *Terminalia chebula* Retz. (CC_{50} = 235 µg/mL)[10], for which is also reported an antioxidant protective effect related to the hydrolysable tannins (ellagic acid, gallic acid) and flavonoids (flavonol aglycones) of its fruit extract[11].

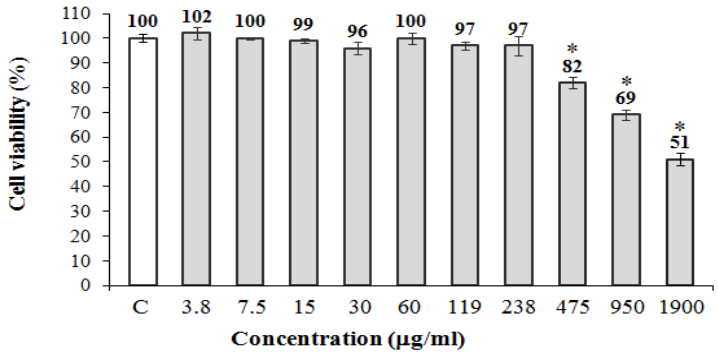


Fig.1. Evaluation of cytotoxicity of methanol 80% of *T. laxiflora* leaves extract in Vero cells after 72h of incubation at 37°C and 5% CO_2 by the MTT assay. Control: without polysaccharide treatment. *Bars* represent means, with *vertical lines* indicating standard deviations, $n = 3$, $*P < 0.01$.

3.2. Antiviral activity studies

The preliminary evaluation of anti-HSV-1 activity of *T. laxiflora* methanol 80% leaf extract was done at the highest non-toxic concentration, determined by the MTT method. The extract inhibited the development of the HSV-1 induced cytopathic effect (rounded cells and production of syncytium) at this concentration, but the normal morphology of the cells was not preserved, as observed in Fig. 2. Although this alteration did not affect the cellular viability, it has to be better investigated further, because it could be related to some kind of adverse effect of the extract on Vero cells. The extract was tested at concentrations below 238 µg/mL, giving an $EC_{50} = 80 \pm 3$ µg/mL, which was 4 times higher than acyclovir (EC_{50}=20 $\pm$ 1 µg/mL). Similar results were reported for the aqueous extract of *Terminalia chebula* fruits which inhibited different strains of HSV-1 at EC_{50} concentrations ranging from 81.2 µg/mL to 82.0 µg/mL using the plaque reduction assay[10]. The hydrolysable tannin casuarinin extracted from *Terminalia arjuna* presented an EC_{50} = 1.5 µM or 1.4 µg/mL, which was 5 times higher than acyclovir by the plaque reduction method of evaluation of the anti-HSV-2 activity[12].

Even though the methanol 80% extract of *T. laxiflora* leaves has shown a lower anti-HSV-1 activity in comparison to acyclovir and promoted alterations of the Vero cells normal morphology at the inhibitory concentrations, the purified fractions of this extract have potential to present higher activities and perhaps with lower toxicity. It is possible that part of the observed anti-HSV-1 activity of the methanol 80% extract of *T. laxiflora* should be related to its hydrolysable tannins, like gallic acid, whose anti-HSV-1 activity through the inhibition of the virus attachment and penetration to the host cells was already reported[13].

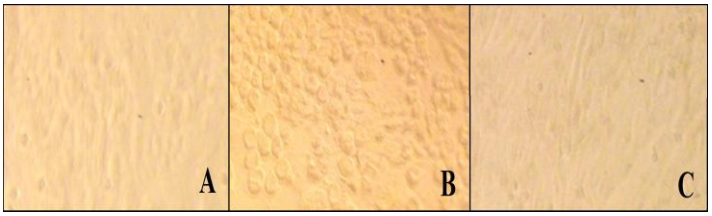


Fig. 2. Screening of the anti-HSV-1 activity in Vero cells after 72h of incubation at 37°C and 5% CO_2 in 96-well microplates by the end point titration method. A: negative control, DMEM 5% FBS; B: positive control, 100x$TCID_{50}$ of HSV-1; C: 100x$TCID_{50}$ of HSV-1 + methanol 80% extract of *T. laxflora* leaves at 238 μg /mL Magnification: 200x.

3.3. Antibacterial activity studies

At all evaluated concentrations (580 and 238 μg/mL), methanol extract of *T. laxiflora* leaves did not inhibit the growth of Gram-positive (*S. aureus*, *S. epidermidis*) or Gram-negative (*E. coli*, *P. aeruginosa*) bacteria (table 1), despite the inhibitory activities of the controls 20% clorexidine (diameters of the zone of inhibition of 28.3 ± 0.6 mm for *S. aureus*, 25.0 ± 0 mm for *S. epidermidis*, 21.0 ± 0 mm for *E. coli*, and 17.7 ± 0.6 mm for *P. aeruginosa*) and 0.005% enrofloxacine (diameters of the zone of inhibition of 31.3 ± 0.6 mm for *S. aureus*, 33.7 ± 0.6 mm for *S. epidermidis*, 36.0 ± 0 mm for *E. coli*, and 21.0 ± 1.0 mm for *P. aeruginosa*). Concentrations above 580 μg/mL were not evaluated due to the cytotoxicity observed in Vero cells, but they could be tested further aiming applications of the extract as antiseptic or disinfectant. In fact, an inhibition of the growth of *S. aureus*, *E. coli* and *P. aeruginosa* strains was reported for the aqueous, ethanol and acetone extracts of *T. chebula* fruits when disks impregnated with 10 mg of the extracts were employed in the disk-diffusion method[14]. Also, the aqueous and ethanol extracts of *T. laxiflora* root bark showed antibacterial activities against diverse Gram-negative bacteria using the agar-diffusion method with concentrations ranging from 53.6 to 75.0 mg/mL[6].

3.4. Phytochemical analysis

Phytochemical analysis of methanol 80% extract of *T. laxiflora* leaves revealed that it contained carbohydrates, tannins, flavonoids, alkaloids and triterpenes (table 2). Chromatographic separation and purification of the methanol extract allowed the identification of nine bioactive compounds: β-sitosterol, m-gallate, gallic acid, ellagic acid and five flavonoids, quercetin, vitexin, isovitexin, quercetin 3-O-α-rhamnoside and rutin (Figure 3). Their structures were elucidated on the basis of UV, ^{1}H-NMR, ^{13}C-NMR and MS analyses.

Table 1: Evaluation of the antibacterial activity of the methanol extract of *T. laxiflora* leaves against *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* by the disk-diffusion method on Mueller-Hinton Agar plates.

Extract/Bacteria	*S. aureus (diameter of the zone of inhition, mm, mean ± standard deviation)*	*S. epidermidis (diameter of the zone of inhition, mm, mean ± standard deviation)*	*E. coli (diameter of the zone of inhition, mm, mean ± standard deviation)*	*P. aeruginosa (diameter of the zone of inhition, mm, mean ± standard deviation)*
T. laxiflora leaves methanol 80% extract	0	0	0	0
20% Clorexidine	28.3 ± 0.6	25.0 ± 0	21.0 ± 0	17.7 ± 0.6
0.005% Enrofloxacine	31.3 ± 0.6	33.7 ± 0.6	36.0 ± 0	21.0 ± 1.0

Table 2: Phytochemical Analysis from *T. laxiflora* leaves methanol 80% extract

Constituents	*Methanol 80% extract*
Triterpenes and /or Sterols	+
Carbohydrates and/or glycosides	+
Flavonoids	+
Coumarins	-
Alkaloids and/or nitrogenous compounds	+
Tannins	+
Saponins	-

(+) presence of constituents, (-) absence of constituents

3.4.1. Observed spectral values of the isolated compounds

β-sitosterol (1)**:** White needles, ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.37 (IH, m, H-6), 3.52 (IH, m, H-3) , 1.09 (3H, s, CH_3-19), 0.98 (3H, d, *J*= 6.5, CH_3-21), 0.92 (3H, t, *J*= 7.4, CH_3-29), 0.85 (3H, d, *J*= 6.7Hz, CH_3-26), 0.81 (3H, d, *J*= 6.7Hz, CH_3-27), 0.75 (3H, s, CH_3-18). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 140.4 (C-5), 121.5 (C-6), 71.6 (C-3), 57.2 (C-17), 56.4 (C-14), 50.3 (C-9), 46.3 (C-24), 42,8 (C-13, 4), 39.8 (C-12), 37.6 (C-1), 36.7 (C-10), 35.9 (C-20), 34.2 (C-22), 31.7 (C-8, 7), 31.4 (C-2), 29.2 (C-25), 28.4 (C-16), 26.2 (C-23), 24.5 (C-15), 23.4 (C-28), 21.1 (C-11), 19.8 (C-26), 19.5 (C-19), 19.2 (C-27), 18.6 (C-21).

Methyl gallate (2): White amorphous powder. UV λmax (MeOH): 272. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 6.9 (2H, s, H-2,6), 3.6 (3H, s, -OCH_3). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 166.8 (-COO), 146.2 (C-3,5), 138.9 (C-4), 119.8 (C-1), 109.5 (C-2,6), 52.3 (-OCH_3).

Gallic acid (3): White amorphous powder. UV λmax (MeOH): 270. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.1 (2H, s, H-2,6). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 166.9 (-COOH), 145.4 (C-3, 5), 137.8 (C-4), 121.4 (C-1), 109.6 (C-2, 6).

Ellagic acid (4): White amorphous powder. UV (λmax, nm): 252, 360. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.3 (2H, s, H-4, 9). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 158.2 (5, 10-CO), 147.8 (C-3,8), 139.7 (C-2,7), 136.7 (C-1a,6a), 112.5 (C-4b,9b), 110.7 (C-4, 9), 107.3 (4a, 9a).

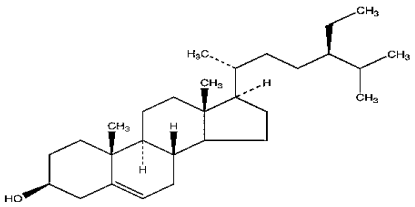
Quercetin (5): Yellow powder. UV λmax (MeOH): 255, 267, 371; (NaOMe): 270, 320, 420; ($AlCl_3$): 270, 455; ($AlCl_3$/HCl): 264, 303sh, 315sh, 428; (NaOAc): 257, 274, 318, 383; (NaOAc/H_3BO_3): 259, 387. EI-MS: m/z 302.

Vitexin (Apigenin 8-C-β-glucopyranoside) (6): Yellow power. UV λmax (MeOH): 271, 339; (NaOMe): 278, 392; ($AlCl_3$): 275, 303 sh, 349; ($AlCl_3$/HCl): 269, 350; (NaOAc): 279, 372; (NaOAc/H_3BO_3): 269, 345. ^{1}H-NMR (DMSO-d6, 400 MHz) δ 13.12 (1H, s, 5-OH), 7.92 (2H, d, *J* = 8.9Hz, H-2′,6′), 6.85 (2H, d, *J* = 8.9 Hz, H-3′,5′), 6.72 (1H, s, H-6), 6.24 (1H, s, H-3), 4.64 (1H, d, *J* =10 Hz, H-1″), 3.2-3.9 (rest of sugar protons, H-2″-6″)**.** (-) ESI-MS: m/z 431 [M-H]$^-$.

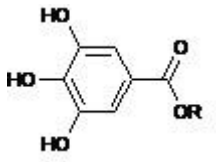
Isovitexin (Apigenin 6-C-β-glucopyranoside) (7): Yellow amorphous power. UV λmax (MeOH):272, 334; (NaOMe): 275, 331sh, 399; ($AlCl_3$): 271, 304, 353, 383; ($AlCl_3$/HCl): 271, 304, 345, 381; (NaOAc): 278, 395; (NaOAc/H_3BO_3):275, 336. ^{1}H-NMR (DMSO-d6, 400 MHz) δ 7.94 (2H, d, *J* = 8.5 Hz, H-2′,6′), 6.89 (2H, d, *J* = 8.5 Hz, H-3′,5′), 6.75 (1H, s, H-6), 6.54 (1H, s, H-3), 4.62 (1H, d, *J* =10 Hz, H-1″), 3.2-3.9 (rest of sugar protons, H-2″-6″)**.** (-) ESI-MS: m/z 431 [M-H]$^-$.

Quercetin 3-O-α-rhamnoside(8): Yellow crystals. ^{1}H–NMR (DMSO–d_6, 400 MHz) δ ppm 7.26 (2H, m, H–2`, 6`), 6.83 (1H, d, *J*=9 Hz, H–5'), 6.49 (1H,d, *J*=2.5 Hz, H–8),6.14 (1H, d, *J*=2.5Hz, H–6), 5.25 (1H, br s, H–1") 0.78 (3H, d, *J*=6Hz). ^{13}C–NMR (100 MHz, DMSO–d_6): δ ppm 177.42 (C–4), 167.45 (C–7), 161.40 (C–5), 157.01 (C–2), 157 (C–9), 149.19 (C–4`), 145.57 (C–3`), 134.12 (C–3), 131.97 (C–6`), 121.40 (C–1`), 115.71 (C–2`), 115.40 (C–5`), 103.10 (C–10), 101.97 (C–1``), 99.98 (C–6), 94.47 (C–8), 71.47 (C–4"), 70.94, 70.85, 70.62 (C–2``, C–5``, C–3"), 17.78 (C6").

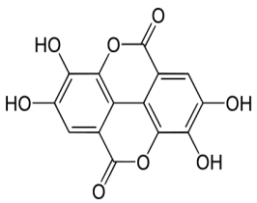
Quercetin 3-O-rutinoside (Rutin) (9): Yellow powder: UV λmax (MeOH): 258, 269, 361; (NaOMe): 276, 322, 416; ($AlCl_3$): 232, 276, 302, 366; ($AlCl_3$/HCl): 232, 276, 302, 366; (NaOAc): 284, 306, 381; (NaOAc/H_3BO_3): 261, 312, 376. ^{1}H-NMR (400 MHz, DMSO-d6): δ ppm 7.54 (2H, m, H-2', 6'), 6.85 (1H, d, *J* = 9 Hz, H-5'), 6.38 (1H, d, *J* = 2.5Hz, H-8), 6.19 (1H, *J* = 2.5 Hz, H-6), 5.35 (1H, d, *J* = 7.5 Hz, H-1"), 4.39 (1H, s, H-1'"), 3.90-3.20 (m, remaining sugar protons), 0.99 (3H, d, *J* = 6 Hz, H-6'"). ^{13}C-NMR (100 MHz, DMSO-d6): δ ppm 177.85 (C-4), 164.70 (C-7), 161.68 (C-5), 157.14 (C-2), 156.95 (C-9), 148.92 (C-4'), 145.25 (C-3'), 133.76 (C-3), 122.12 (C-6'), 121.66 (C-1'), 116.73 (C-2'), 115.72 (C-5'), 104.41 (C-10), 101.66 (C-1'"), 101.23 (C-1"), 99.24 (C-6), 94.16 (C-8), 74.58 (C-3"), 72.33 (C-5"), 72.2 (C-4'"), 71.05 (C-2"), 70.8 (C-2'"), 70.87 (C-3'"), 70.49 (C-4"), 63.74 (C-6"), 18.19 (C-6'").



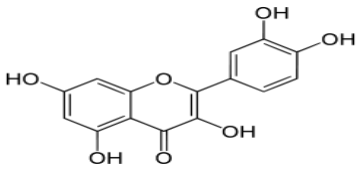
1. β-Sitosterol



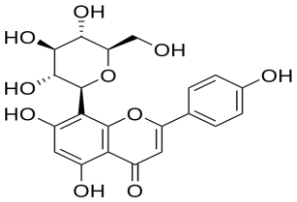
2. Methyl gallate (R=CH_3)
3. Gallic acid (R=H)



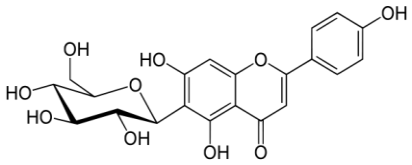
4. Ellagic acid



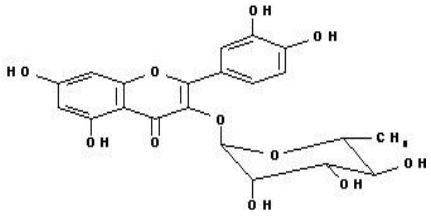
5.Quercetin



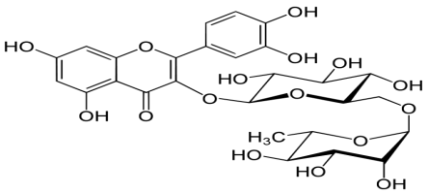
6. Vitexin



7.Isovitexin



8. Quercetin 3-O-α-rhamnoside



9.Quercetin 3-O-rutinoside (Rutin)

Fig. 3: Chemical compounds isolated from *T. laxiflora* leaves methanol 80% extract

Chromatographic separation and purification of the methanol 80% extract of *T. laxiflora* leaves resulted in the isolation and identification of compound 1 (β-sitosterol) which gave dark spot under short UV light that changed to violet colour on spraying with vanillin sulphuric and heating in an oven at 110°C for 5 min. NMR spectral data has shown signals very close to compound 1 (β-sitosterol), also it is identified by other authors[15]. The three hydrolysable tannins, compound 2 (methyl gallate), compound 3 (gallic acid) and compound 4 (ellagic acid) gave specific colour reaction with $FeCl_3$ and its NMR spectral data are very close to that of Nawwar[16]. Compound 5 (quercetin) yellow spot and gave fluorescence yellow colour after spraying with $AlCl_3$ and its spectral data are very similar to Lawrence[17].

Compound 6 (vitexin) and compound 7 (isoviotexin), each compound gave deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$, with complete acid hydrolysis, there is no change for compounds 6 and 7 and thus, the compounds 6 and 7 were subjected to ferric chloride degradation and the products being co-paper chromatography with authentic flavonoid aglycone and sugar samples, where apigenin as an aglycone and glucose moiety were detected and all spectral data of both compounds were very close to that of Yun-Lian[18]. Compound 8 (quercetin 3-*O*-α-rhamnoside) which gave deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$ and complete acid hydrolysis gave quercetin as an aglycone and rhamnose as sugar moiety and its spectral data was very similar to that described by Lawrence[17].

Compound 9 (rutin) gave a deep purple spot under UV light and changed to yellow when subjected to ammonia and $AlCl_3$ and complete acid hydrolysis gave quercetin as an aglycone and glucose and rhamnose as sugar moieties and its spectral data was very close to that of Biruk[19]**.** All the chemical structures of beta-sitosterol, three hydrolysable tannins (methyl gallate, gallic acid and ellagic acid), other compounds, flavonoid aglycones and their sugars, were established by comparison of their spectral data with those reported in literature[20].

4. CONCLUSION

This work highlight the importance of natural sources from plant origin in controlling viral diseases and this study will pave a way for the treatment pattern of HSV-1 infection and potential exploitation of natural wealth for screening of antiviral principles present in methanol 80% extract of *T. laxiflora* leaves.

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