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**Journal of Chemical and Pharmaceutical Research, 2015, 7(5):29-43**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Phytochemical and biological study of *Albizia lebbeck* stem bark

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**ABSTRACT**

*Bioassay guided phytochemical investigation of petroleum ether, chloroform and ethyl acetate fractions obtained from biologically active total alcoholic extract of stem bark of Albizia lebbeck L. Benth led to isolation of nine known compounds including lupenone (1), freidelin (2), lupeol (3), sapiol (4), mixture of β-sitosterol & stigmasterol (5), β sitosterol- 3-O-glucoside & stigmasterol -3-O-glucoside mixture (6), stigmasterol -3-O-glucoside (7), luteolin (8) and rutin (9). Their structures were established on the basis of spectroscopic methods including UV, MS, IR, $^{1}H$ &$^{13}C$ NMR, $^{1}H$ $^{1}H$ COSY and through comparison with published data and authentics. Compounds **6-8** were isolated for the first time from A. lebbeck. Qualitative estimation of the phenolic and flavonoidal contents of different extracts showed that the plant is rich in phenolic and flavonoidal contents. The major phenolic and flavonoid compounds were detected in ethyl acetate fraction and identified as e-vanillic acid (15079.44 ppm), luteolin (6024.92 ppm) and quercetrin (3120.15 ppm) respectively. GLC/MS analysis of unsaponifiable matter of A. lebbeck, revealed nineteen compounds constituting 55.8% of the total unsaponifiable matter were identified. In addition the GLC analysis of fatty acid methyl esters revealed the presence of 13 fatty acids, ten of which were identified and constitute 91.44 %. Moreover the different A. Lebbeck stem bark extracts showed variable promising strengths in anti-inflammatory, analgesic, antioxidant, cytotoxic and antimicrobial activities.*

**Keywords**: *A. lebbeck* stem bark, flavonoids, sterols, triterpenes, analgesic, anti-inflammatory, antimicrobial, cytotoxic.

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## INTRODUCTION

Genus *Albizia* (Fabaceae) comprises approximately 150 species, mostly trees and shrubs native to tropical and subtropical regions of Asia and Africa [1]. *A. lebbeck* is a member of this genus and used in folk medicine to treat inflammatory conditions as asthma, arthritis, burns allergic rhinitis, bronchitis and leprosy [2] and it have been claimed to be useful in treatment of Alzheimer`s and Parkinson`s diseases [1]. Moreover the extracts of *A. lebbeck* exhibited versatile biological effects as antioxidants [3], hepatoprotective, cardiotonic, lipid-lowering, hypoglycemic activities [4,5] antihistaminic [2] and antimicrobial [6]. Literature survey on *A. lebbeck* revealed the presence of sterols and triterpenes [7], phenolic compounds, flavonoids [8], isoflavone [9], alkaloids [10], miscellaneous compounds [11] and saponins [12]. But there is no report about *A. lebbeck* plant growing in Egypt except the isolation of kaempferol and quercetin-3-*O*-*α*-rhamnopyranosyl-(1-6)-*β*-glucopyranosyl-(1-6)-*β*-galactopyranoside from the leaves [13], this prompted us to investigate this plant. The present work deals with the isolation and identification of nine known compounds **1-9**; compounds **6-8** were isolated for the first time from *A. lebbeck*, in addition to quantitative and qualitative estimation of flavonoidal and phenolic contents of *A. lebbeck* extracts. The

analgesic, anti-inflammatory, antioxidant, cytotoxic and antimicrobial activities of different *A. lebbeck* stem bark extracts were also evaluated.

## EXPERIMENTAL SECTION

***General experimental section***

Büchi rotatory evaporator was used for evaporation of solvent; Melting point were determined by using Digital, electro-thermal LTD (England) apparatus; GL-58 ($\lambda_{max}$ 254 and 365 nm) UV lamp was used for TLC visualization UVP; Circulating hot air oven W.T-binder 7200 (Germany) was used for drying and activation of silica gel plates; Shimadzu UV-1700 spectrophotometer (Japan) was used for UV spectral analysis of flavonoids. Infrared spectral analysis were recorded in potassium bromide disks on a PyeUnicam SP 3000 and IR spectrophotometer, Jasko, FT/IR-460 plus. GC-MS analysis was carried out on: Shimadzu GC-MS-QP5050A mass spectrometer at 70 e.V. Bruker Daltonics flex analysis; acetonitrile : $H_2O$ (1:5) was used as a matrix for ESI-mass.$^1H$ & $^{13}C$ NMR spectral analyses were obtained by: JEOL at 500, 125 MHz., Bruker at 400 MHz and GEMINI at 300, 75 MHz. Chemical shifts were given in ppm with the TMS as internal standard. GLC analysis of the total fatty acids methyl esters were carried out on a PyeUnicam Series 304 gas chromatograph: Detector (Dual flame ionization detector), Temp. of detector (300$^o$C), Recorder (Dual channel recorder), Temp. of injector (250$^o$C), Column temp [70 to 190 $^o$C ; 8$^o$C/min] then isothermally at 190$^o$C for 25 min; Column package [Diatomite C; 100-120 mesh], Liquid phase 10 % PEGA, Column dimensions (1.5 m × 4 mm), Nitrogen flow rate (30 ml / min.); GLC/MS analysis of the unsaponifiable matter was carried out on GC MODEL: TRACE GC 2000 produced by THERMO and Mass spectrophotometer Model: SSQ 7000 produced by FINNIGAN**;** HPLC analysis of phenolics and flavonoids were carried out on HPLC apparatus (Agilant 1100 series equipped with autosampler) using gradient elution with 2.5% $CH_3COOH$ in $H_2O$/ acetonitrile as mobile phase.

***Chromatographic Solvent Systems:***

The following solvent systems were used in TLC development

I. Light petroleum : chloroform (1:1)
II. Light petroleum : chloroform : methanol (15 : 15 : 1)
III. Light petroleum : chloroform : methanol (15 : 15 : 2)
IV. Ethyl acetate : methanol : $H_2O$ ( 6 : 1 : 0.8)
V. Ethyl acetate : methanol : $H_2O$ (6 : 2 : 0.8)

***Plant material***

The plant material used in this work, *Albizia lebbeck* L. Benth family Fabaceae, was collected in the flowering stage on May 2010 from the vicinity of Benha governorate, Qalioubia, Egypt. The identification was verified by Prof. Dr. Hussain Abdel Baset Professor of Botany, Faculty of science, Zagazig University, Egypt. A voucher specimen is deposited in Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt. The plant was shade dried and ground to moderately fine powder.

**Extraction and isolation:**

TLC investigation using silica gel $GF_{254}$ chromatoplates, for petroleum ether extract of *A. lebbeck* stem bark revealed the presence of five major spots.

***Isolation of compounds 1-5 from petroleum ether fraction of stem bark***

About 15 gm of petroleum ether fraction was placed on the top of silica gel column. The elution was started with light petroleum then the polarity was increased gradually using dichloromethane and methanol respectively, the collected fractions were concentrated under reduced pressure, examined by TLC using solvent system (I, III) and similar fractions were combined. Compound **1** (62 mg) was isolated from fractions eluted with 20% $CH_2Cl_2$/light pet. Fractions eluted with 20% $CH_2Cl_2$ / light pet. afforded 49 mg of white needles of compound **2**. In addition fractions eluted with 30% $CH_2CL_2$ / light Pet; 40% $CH_2CL_2$/light Pet; and 65 % $CH_2CL_2$ / light pet. yielded compounds **3** (10 mg), **4** (20 mg) , **5** (69 mg of **5a** and **5 b** as a mixture) respectively.

***Isolation of compounds 6 and 7 from chloroform fraction of the stem bark***

About 20 gm of the chloroform soluble fraction was subjected to silica gel column elution started with light petroleum and the polarity was increased gradually using dichloromethane then methanol. Fractions eluted with 3%

and 4% MeOH / $CH_2Cl_2$ were separately collected and concentrated, then the resulting residues were subjected to crystallization from hot methanol to afford compounds **6** (440 mg) and **7** (328 mg).

***Isolation of compounds 8 and 9 from ethyl acetate fraction of the stem bark***

About 11.6 gm of ethyl acetate soluble fraction of the stem bark was subjected to silica gel flash column eluted with (light petroleum & dichloromethane; 1:1) and the polarity was increased gradually using dichloromethane then methanol. Twelve fractions, 1000 ml each, were collected. The important fractions were subjected to sephadex $LH_{20}$ column eluted with methanol to give compounds **8** (7 mg) and **9** (17 mg).

*Compound **1***: (62 mg), white needles; $R_f$ value 0.44 (solvent system I); mp 164-166$^0$C; EI-MS *m/z*: 424 [$M^+$], 409, 381, 313, 218, 205, 204, 189, 161, 149, 135; IR $\nu_{max.}$ (KBr) cm$^{-1}$: 2939 and 2861 (C-H), 1705 (C=O), 1643 (C=C), 1454 ($CH_2$) and 1383 ($CH_3$); $^1$H-NMR ($CDCl_3$, 400 MHz): $\delta_H$ 1.12 (3H, s, $CH_3$-23), 1.09 (3H, s, $CH_3$-24), 0.97 (3H, s, $CH_3$ -25), 0.95 (3H, s, $CH_3$-26), 0.81 (3H, s, $CH_3$-27), 1.04 (3H, s $CH_3$- 28), 4.59, 4.71 (2H, $CH_2$-29) and 1.09 (6H, s $CH_3$-30 and $CH_3$-24).

*Compound **2***: (49 mg) of white needles with mp 257 - 259 $^0$C; $R_f$ value 0.37 (solvent system I); IR $\nu_{max.}$ (KBr) cm$^{-1}$: 2923 and 2857 (C-H), 1712 (C=O), 1459 ($CH_2$) and 1310 ($CH_3$ ); The $^1$H-NMR ( $CDCl_3$, 300 MHz): $\delta_H$ 0.87 (d, *J*= 6.3 Hz , H-23), 0.72 (3H, s, $CH_3$-24) , 0.80 (3H, s, $CH_3$-25), 0.95 (6H, s, $CH_3$-26 and $CH_3$-30), 1.03 (3H, s, $CH_3$-27), 1.18 (3H, s, $CH_3$-28), 0.93 (3H, s, $CH_3$-29); the $^{13}$C-NMR ($CDCl_3$, 125 MHz): $\delta_C$ 22.2 (C-1), 41.5 (C-2), 213.3 (C-3), 58.1 (C-4), 42.1 (C-5) , 41.2 (C-6), 18.6 (C-7), 52.9 (C-8), 37.3 (C-9), 59.3 (C-10), 35.5 (C-11), 30.4 (C-12), 38.2 (C-13), 39.9 (C-14), 32.3 (C-15), 36.8 (C-16), 29.9 (C-17), 42.9 (C-18), 35.4 (C-19), 28.1 (C-20), 32.7 (C-21), 39.5 (C-22), 16.8 (C-23), 14.6 (C-24), 18.2 (C-25), 20.2 (C-26), 19.2 (C-27), 32.0 (C-28), 31.7 (C-29), 35.2 (C-30); EI-MS *m/z*: 426 [$M^+$], 411, 341, 302, 287, 275, 273, 257, 246, 232, 230, 218, 205, 190, 179, 152, 150, 148, 137, 134, 123, 109, 81, 69 and 55.

*Compound **3***: (10 mg) white crystals with mp 214 - 216 $^0$C; $R_f$ value 0.67 (solvent system II; IR $V_{max}$ (KBr) cm$^{-1}$: 3409 (O-H), 2926 and 2857 (C-H ), 1637 (C=C), 1459 ($CH_2$ ), 1378 ($CH_3$) and 1142 (C-O); EI-MS *m/z*: 426 [$M^+$], 411, 393, 299, 297, 257, 247, 229, 231, 218, 207, 203, 189, 177, 161, 121 and 93.

*Compound **4***: (20 mg) white flakes with mp 92 - 94$^0$C; $R_f$ value 0.62 (solvent system II); IR $\nu_{max.}$ (KBr) cm$^{-1}$: 3298 (O-H), 2919 and 2848 (C-H), 1476 ($CH_2$); $^1$H-NMR ($CDCl_3$ , 300 MHz ): $\delta_H$ 3.67 (2H, t, *J*=4.8 Hz H-1) , 1.57 (H-2), 1.44 (H-3), 1.32 : 1.20 (H-4-33), 0.88 (3H, t, *J*= 6.3 Hz, H-34); $^{13}$C-NMR ($CDCl_3$, 75 MHz ) ; 63.1 (C-1), 32.8 (C-2), 31.6 (C-3), 29.6 -22.6 (C-4-33), 14.1 (C-34); EI-MS *m/z*: 494 [$M^+$], 448, 421, 392, 378, 365, 350, 337, 308, 279, 251, 237, 223, 209 , 195, 181, 167, 153, 138, 125, 111, 82, 68 and 57.

*Compounds **5*** (***5a** and **5b***): (69 mg) white flakes with m.p. 137 - 139°C; $R_f$ value 0.48 (solvent system II); IR $\nu_{max}$ (KBr) cm$^{-1}$: 3431(O-H), 2931 and 2863 (C-H), 1648 (C=C), 1458 ($CH_2$), 1040 (C-O); EI-MS *m/z*: 414 [$M^+$], 412 [$M^+$], 398, 370 , 301, 300, 271, 257, 255, 215, 214, 213, 185, 173, 160, 145, 133, 109, 107, 105, 95, 83, 81, 67 and 55.

*Compound **6***: (440 mg), white crystals with mp 272-274$^0$C; $R_f$ value 0.56 (solvent system III). IR $\nu_{max.}$ (KBr) cm$^{-1}$: 3417 (O-H), 2941 and 2871 (C-H), 1645 (C=C), 1456 ($CH_2$), 1262 ($CH_3$ ), 1047 and 841. The EI-MS *m/z*: 577 [$M_1^+$], 575 [$M_2^+$], 414 [$M_1^+$], 412 ($M_2^+$-sugar), 398, 397, 396, 394, 382, 381, 303, 273, 255, 213, 168, 173, 161, 145, 133, 97, 95, 83, 81, 69, 57 and 55.

*Compound **7***: (30 mg), white amorphous powder, m.p. 288 – 290$^0$C and $R_f$ value 0.4 (solvent system III); IR $\nu_{max.}$ (KBr) cm$^{-1}$: 3426 (O-H), 2936 and 2880 (C-H), 1634 (C=C), 1450 ($CH_2$), 1374 ($CH_3$), 1067, 1034 and 888; EI-MS *m/z*: 575 [$M^+$ +H], 412 ($M^+$ - sugar), 395 , 380 , 329 , 351, 325, 298 , 273, 255, 213, 161, 146, 135, 98, 95, 70, 58 and 55.

*Compound **8*** : (70 mg), yellow amorphous powder; m.p. 325°C; $R_f$ value of 0.74 in solvent system IV; UV $\lambda_{max}$ (MeOH) 254, 349, (MeOH + NaOM) 266, 401, (MeOH + $AlCl_3$) 273, 421, (MeOH + $AlCl_3$ + HCl) 274, 355, (MeOH + NaOAc) 269, 393(MeOH + NaOAc + $H_3BO_3$) 264, 376; IR $V_{max}$ (KBr) cm$^{-1}$: 3420 (O-H), 2943 and 2859(C-H), 1609 (C=O), 1509 (C=C), 1261, 1168, 1120 (C-O), 830, 755, 688; EI-MS *m/z* : 286 [$M^+$, 82.3], 258, 228, 153,135, 134, 96; The $^1$H -NMR ($CD_3OD$ , 500 MHz): $\delta_H$ 6.52 (1H, s, H-3), 6.18 (1H, d, *J*=1.75 Hz, H-6), 6.42 (1H, d, *J*=1.75 Hz, H-8), 7.36 (1H, d, *J*= 2 Hz, H-2`), 6.98 (1H, d, *J*= 8.6 Hz, H-5`) and 7.34 (1H, dd, *J*= 2, 8.6 Hz,

H-6`); $^{13}$C-NMR ($CD_3OD$, 125 MHz): $\delta_C$ 160.0 (C-2), 103.1 (C-3), 182.5 (C-4), 158.1 (C-5), 101.9 (C-6), 165.0 (C-7), 101.8 (C-8), 164.7 (C-9), 103.0 (C-10), 118.5 (C-1`), 112.4 (C-2`), 145.7 (C-3`), 149.6 (C-4`), 115.9 (C- 5`) and 122.3 (C-6`).

*Compound* ***9***: (17 mg), yellow amorphous powder; mp 214-216$^0$C; $R_f$ value of 0.49 in solvent systems V; UV $\lambda_{max}$ (MeOH) 258, 358, (MeOH + NaOMe) 272, 409 (MeOH + $AlCl_3$) 273, 427, (MeOH + $AlCl_3$ + HCl) 256, 358, (MeOH + NaOAc) 266, 384, (MeOH + NaOAc + $H_3BO_3$) 263, 378; IR $V_{max}$ (KBr) cm$^{-1}$: 3374 (O-H), 2925 (C-H aromatic), 1653 (C=O), 1605 (C=C), 1501(C=C ), 1358, 1298, 1065 (C-O); The $^1$H -NMR ($CD_3OD$ , 400 MHz): $\delta_H$ 6.21 (1H, brs H-6), 6.41 (1H, brs, H-8), 7.80 (1H, brs H-2`), 6.90 (1H, d, *J*= 8.4 Hz, H-5`) and 7.60 (1H, d, *J*= 8.4 Hz, H-6`), 5.10 (1H, d, *J*=7.6 Hz, H-1``), 3.46-3.53 (6H, m, 2``-H6``), 4.50 (1H, brs H-1``), 3.46-3.53 (4H, m, 2``-H5``) and 1.30 (3H, brs, H-6``) ; EI-MS *m/z* 302 [$M^+$], 286, 152, 150, 137, 135, 124, 118, 108, 96 and 57.

***Acid hydrolysis of compounds 6 and 7***

Acid hydrolysis of compounds **6** and **7** was carried out and afforded glucose as the sugar residue which confirmed by co-chromatography with authentic sample.

**GLC analysis of the fatty acids constituent and GLC/MS analysis of the unsaponifiable matter of the stem bark of *A. lebbeck*:**

***Saponification of light petroleum soluble fraction of the stem bark***

About 5.0 g of light petroleum soluble fraction of the stem bark was subjected to saponification process according to the method mentioned previously [14,15] to give 900 mg of unsaponifiable matter (USM) and 700 mg of fatty acids content**.**

***Preparation of fatty acid methyl esters***

Methylation of about 200 mg of fatty acids residue was carried out according to the previously reported method [15,16] to afford about 210 mg of fatty acids methyl esters.

***GLC analysis of fatty acid methyl esters***

Gas liquid chromatography analysis of the fatty acids methyl esters was carried out against references of methyl esters of many fatty acids including capric, lauric, myristic , palmitic, palmitoleic, margaric, stearic, oleic and linoleic and arachidic. Identification of fatty acids methyl esters was carried out by comparison of the retention times of the fatty acid methyl esters with that of the authentic samples. The quantitative estimation was carried out by the peaks area measurement and the results were recorded in table (1).

***Analysis of the unsaponifiable matter***

Analysis of the unsaponifiable matter was performed using GLC/MS. Identification of the different peaks were done by comparing the mass fragments of the isolated peaks with those of library reference (Wiley) and literature published data [15]. The results are summarized in table (2).

**Qualitative and quantitative estimation of total phenolic and flavonoid contents of *A. lebbeck stem bark* extracts:**

***Quantitative estimation of the total phenolic contents of A. lebbeck stem bark extracts***

Spectrophotometric determination of the total phenolic content was carried out according to procedure reported in the European Pharmacopoeia, using the Folin-Ciocalteu colorimetric method [17-19]. Total phenolics were expressed as mg of gallic acid equivalents (mg GAE) /g of the extract.

***Preparation of gallic acid standard calibration curve***

The standard stock solution was prepared by dissolving 30 mg of gallic acid in 100 ml distilled water at a final concentration of 300 µg/ml. This stock solution was serially diluted with distilled water to obtain the required concentration (equivalent to 1- 300 µg/ml). An aliquot (0.5 ml) of each standard solution was mixed with 0.5 ml distilled water, 5 ml of Folin-Ciocalteu reagent (a mixture of phosphomolybdate and posphotungstate) and 4 ml of saturated sodium carbonate (75 gm/L). The absorbance of the resulting solution (blue) was measured after incubation for 2 hrs at $\lambda_{max}$ 765 nm (using a UV spectrophotometer) against blank similarly prepared except for replacing test solution by 0.5 ml distilled water. All sample manipulations were performed protected from light. For each concentration, four replicates were carried out and the average of the obtained absorbance was plotted versus the concentration (figure 1).

***Estimation of the phenolic contents***

The total alcoholic extract and different fractions of *A. lebbeck* were dissolved in methanol at a concentration of 4 mg/ml. Each test solution was treated as mentioned before for the standard solution then the phenolic contents calculated as gallic acid was deduced from the pre-established calibration curve in figure 1, and the results were obtained and recorded in table (3).

***Quantitative estimation of the total flavonoids content of A. lebbeck stem bark extracts***

Spectrophotometric determination of the total flavonoid contents of the total alcoholic, ethyl acetate and butanol extracts of stem bark were carried out according to procedure that was modified from the method reported by Woisky and Nabavi [20, 21] using the aluminum chloride for determination of the flavonoids contents. Total flavonoids were expressed as mg of quercetin equivalents (mg quercetin/g of extract) and as mg of rutin equivalents (mg rutin/g of the extract).

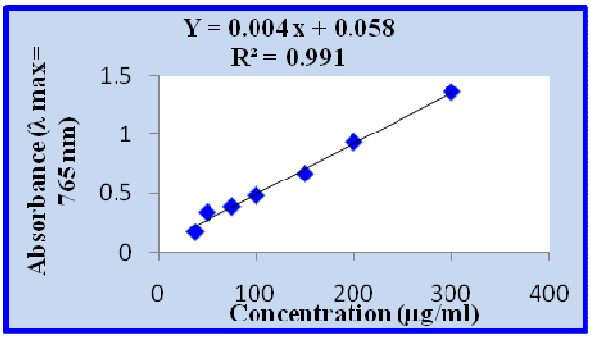


**Figure 1: Calibration curve for standard gallic acid**

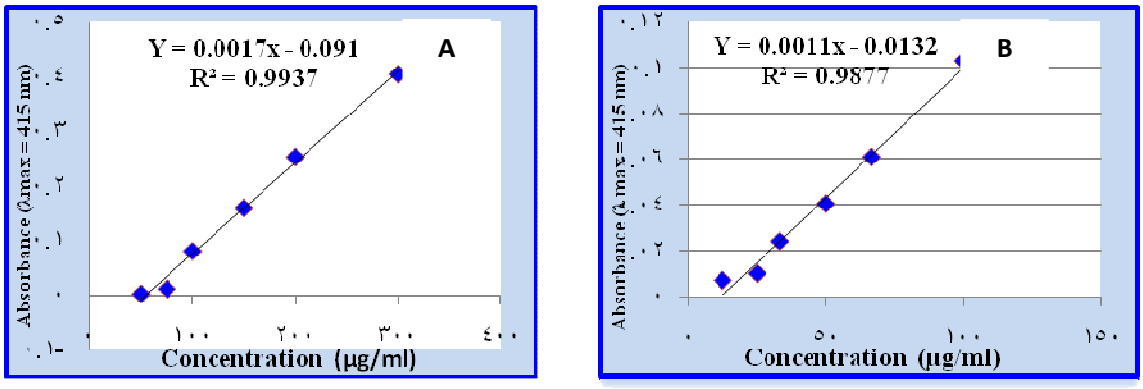


**Figure 2: Calibration curves for standard quercetin (A) and rutin (B)**

***Preparation of quercetin and rutin standard calibration curves***

The standard stock solution was prepared by dissolving 30 mg of standard quercetin and 10 mg of standard rutin in 100 ml of 95% ethanol at a final concentration of 300 µg/ml and 100 µg/ml respectively. This stock solution was serially diluted with 95 % ethanol to obtain the required concentrations (equivalent to 50- 300 µg/ml and 12.5-100 µg/ml respectively). An aliquot (0.5 ml) of each standard solution was mixed with 1.5 ml 95% ethanol, 0.1 ml of 10 % (w/v) aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The absorbance of the resulting solution (yellow) was measured after incubation for 30 minutes at $\lambda_{max}$ 415 nm (using a UV spectrophotometer) against blank similarly prepared except for replacing aluminum chloride by the same volume of distilled water. All sample manipulations were performed protected from light. For each concentration, four replicates were carried out and the average of the obtained absorbance was plotted versus the concentration. The results were represented in figure (2).

***Estimation of the total flavonoidal content of A. lebbeck stem bark extracts***

The total alcoholic extract and different fractions of *A. lebbeck* were dissolved in 95% ethanol at a concentration of 2 mg/ml. Each test solution was treated as mentioned before for the standard solution. The flavonoid contents calculated as quercetin (flavonoidal aglycone) and rutin (flavonoidal glycoside) equivalents were deduced from the pre-established calibration curves in figure (2) and measured as mg quercetin or rutin equivalent/ g extract. The absorbances and flavonoid contents of different fractions were shown in table (4).

***HPLC analysis of phenolic and flavonoidal compounds of A. lebbeck stem bark extracts***

According to the results obtained from the quantitative spectrophotometric analysis of total phenolics and flavonoidal contents, *A. lebbeck* plant is rich in phenolics and flavonoids. Therefore, study of phenolic and flavonoidal components of the total alcoholic extract, ethyl acetate and butanol fractions was carried out to identify their contents using HPLC technique and by the aid of series of standards phenolic and flavonoidal compounds. The results were reported in tables 5 and 6 respectively.

**Biological Studies**

**Biological activities of *A. lebbeck* stem bark extracts**:

Reviewing the current literature for the importance of *A. lebbeck* growing worldwide revealed many biological interests. The species growing in Egypt has not received attention. So the present study was undertaken to study analgesic, anti-inflammatory, antioxidant and antimicrobial activities of different extracts of *A. lebbeck*

***Anti-inflammatory activity***

The anti-inflammatory activity of the total alcoholic extract of the stem bark of *A. lebbeck* on the rat paw edema induced by carrageenan (Sigma Aldrich) was studied using the hind paw oedema method [22]. Diclofenac sodium (Novartis) was used as reference standard. Twenty male albino rats weighing 200–220 gm were used in this study. The Faculty of Veterinary Medicine, Zagazig University, Egypt provided the experimental animals. All animals were held under standard laboratory conditions in the animal house of the Faculty of Pharmacy, Zagazig University at 27°C with 12/12 light-dark cycle. They were fed laboratory diet and water ad libitum. The rats were divided into three groups, five rats in each, the first group was served as control and was given gum acacia solution (7%). The second group received diclofenac sodium at the dose of 4 mg/kg. The third group was given the total alcoholic stem bark extract (120 mg/kg) suspended in 7% gum acacia. All treatments were administered by means of oral administration. Thirty minutes later, paw oedema was induced by subcutaneous injection of 0.1 ml carrageenan (1% suspention in saline) into the sub-plantar surface of the right hind paw of all animals. The left legs of hind paw were injected by 0.1ml normal saline. The hind paw diameter was measured, using a micrometer, just before the injection of carrageenan and 1, 2, 3, 4, 5 and 24 hr after the injection. The hind paw diameter was measured for each rat at each time interval and the mean thickness of oedema was calculated. Since the time course of the effect was followed, it was possible to use the cumulative anti-inflammatory effect during the whole observation period as the area under the curve (AUC). Because the AUC curve represents the integrated anti-inflammatory effect (variation of paw diameter) during the observation period, it then includes both the maximal response and the duration of action. The AUC relating variation of edema to time was obtained using the trapezoidal rule [23]. Total inhibition (TI, %) was obtained for each group and at each record, using the following equation:

$$TI\ (\%) = [AUC\ control - AUC\ treat] \times 100 / AUC\ control.$$

Data were expressed as mean ± standard error of mean (SEM) of five animals.

***Analgesic activity***

***Materials and Methods***

The analgesic activity of the total alcoholic extract of the stem bark of *A. lebbeck* was determined using the acetic acid-induced writhing technique [24]. Acetic acid (0.6% solution in normal saline) 0.1 ml/10 g body weight of was used as an inducer for writhing. 18 Adult male mice weighing 20–25 g were used and Diclofenac sodium (Novartis) was used as reference standard. A sensitivity test for acetic acid was carried out one day before experiment as follows: each mouse was injected intra-peritoneally by 0.1 ml/10 g body weight of 0.6% of the acetic acid. Mice were observed for 15 min.; the response in animals manifested as a contraction of the abdominal muscles and stretching of hind limbs, the mouse that did writhing was considered as positive. After 24 hours of the sensitivity test, acetic-acid-sensitive mice were divided into three groups (n = 6). The first group was given gum acacia mucilage (7%) intra-peritoneal and served as control, the second group received the total extract of the stem bark of

*A. lebbeck*, suspended in 7% gum acacia, at a dose of 120 mg/kg orally and the third group received diclofenac sodium at a dose of 4 mg/kg orally. After one hour, acetic acid was injected and the number of writhes during the following 25 min. period in 5 min. intervals was counted.

***Anti-oxidant activity (DPPH free radical scavenging activity)***

The method of Ratty [25] was used. One hundred µL of each extracts (10 mg extract/10 ml methanol) was added to 3 ml of 0.1 mM DPPH methanolic solution. After incubation period of 30 min. at room temperature, the absorbance was determined against a control at λmax 517 nm. Ascorbic acid was used as a positive control. All the determinations were performed in four replicates and averaged. Percentage of antioxidant activity of free radical DPPH was calculated as follow:

DPPH scavenging activity % = 100-{[($A_\circ$– $A_1$)/$A_\circ$] × 100}

Where $A_\circ$ is the absorbance of the blank sample and $A_1$ is the absorbance of tested extract.

***Antimicrobial activity***

Cup-plate method [26] was used to detect the preliminary antimicrobial activity of the total alcoholic extract, light petroleum and chloroform fractions of stem bark. The samples were dissolved in dimethyl formamide (DMF) at concentration of 100 mg/ml. The nutrient agar or Saburaud's agar were seeded by about $10^6$ microbial cells. Gram +ve bacteria (*Staph. aureus* ATCC 6538, *Staphylococcus aureus* ATCC 12228 and *Staph. epidermidis* ATCC 12228) and Gram –ve bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia. coli* ATCC 10536 and *Escherichia. coli* ATCC25922) as well as fungi (*Aspragillus niger* ATCC 16404 and *Candida albicans* ATCC 10231) are standard strains obtained from the Department of Microbiology, Faculty of Pharmacy, Zagazig University and used as tested microorganisms. Each cup was filled by about 100 µl from each extract (100 mg/ml). Amoxycillin and Amphotericin B (5 mg/ml) were used as standard antibacterial and antifungal, respectively. The plates were incubated overnight at 37˚C for bacteria and at 30˚C for fungi. Zones of inhibition were measured (mm) and recorded in table (10).

***Cytotoxic activity***

HePG-2 (Hepatocarcinoma), HCT-11 6 (Colon carcinoma),HEP-2 (Larynx carcinoma), HELA (Cervical carcinoma) and MCF-7 (Breast carcinoma) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown as mono-layers in growth RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The monolayers of 10,000 cells adhered at the bottom of the wells in a 96-well microtitre plate incubated for 24 hr at 37$^o$C in a humidified incubator with 5% $CO_2$. The monolayers were then washed with sterile phosphate filtered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100µl from different dilutions of the test sample in fresh maintenance medium and incubated at 37$^o$C. A control of untreated cells was made in the absence of the test sample. Six wells were used for each concentration of the test sample. The observation under the inverted microscope was recorded every 24 hr. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and read the absorbance at $\lambda_{max}$ 490 nm using ELISA reader (SunRise TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered as 100 % proliferation. The number of viable cells was determined using ELISA reader as previously mentioned before and the percentage of viability was calculated as:

[1- (ODt / ODc)] x 100 %]

Where, ODt : optical density of wells treated with the test sample.
ODc : optical density of untreated cells

The LD50 value, which reduce the cell number by 50%, was determined from dose response curve.

## RESULTS AND DISCUSSION

**Structure elucidation of the isolated compounds:**

***Compounds 1-3***

The physical and chemical data of compounds **1-3** suggested steroidal or triterpenoidal compounds (Cook 1961), their IR spectra revealed the presence of peaks for ($CH_2$) and ($CH_3$), in addition to sharp peaks around 1700 $cm^{-1}$ (for C=O, compounds **1** and **2**), and 3409 $cm^{-1}$ (for O-H, compound **3**) suggesting saturated steroid or triterpenoid ketone for compounds **1** and **2** and indicating the presence of secondary hydroxyl group in compound **3** ([16, 27].The EI-MS spectra of compounds **1-3** showed molecular ion peaks at *m/z* 424 ($C_{30}H_{48}O$), *m/z* 426 ($C_{30}H_{50}O$) and *m/z* 426 ($C_{30}H_{50}O$) for compounds **1-3** respectively with fragmentation pattern characteristic for saturated pentacyclic triterpenes [28]. By comparing the spectral data of compounds **1-3** with the available literature [12, 29, 30] and direct comparison with authentic samples (mp, Co-TLC), compounds **1-3** were identified as lupenone, friedelin and lupeol respectively. Compounds **1-3** were previously isolated from *A. lebbeck* [12].

***Compound 4***

The IR spectrum of compound **4** revealed the presence of broad peak at 3298 $cm^{-1}$ (-OH), 2919 and 2848 $cm^{-1}$ for ($CH_2$) stretching and 1467 ($CH_2$) bending. The EI-MS spectrum of compound **4** showed a molecular ion peak at *m/z* 494 [$M^+$] corresponding to molecular formula $C_{34}H_{71}O$. The mass peak at *m/z* 448 [$M^+$-18-28]. Losses of several 14 mass units ($CH_2$) revealed the presence of long chain hydrocarbon. By comparing the previously mentioned data with the available literature, compound **4** was proved to be sapiol. According to the available literature, sapiol was isolated before from *A. lebbeck* [11].

***Compounds 5 and 6***

The physical properties and colour reactions of compounds **5** and **6** suggested steroidal or triterpenoidal skeletons [31] IR spectra of compounds **5** and **6** showed that the two compounds are closely related to each other and displayed the presence of absorption bands for bonded (OH) group, together with C-O stretching. Furthermore the mass spectrum of compound **5** showed identical fragmentation pattern for the steroidal nucleus with two distinct parent ions at *m/z* 414 (**5**a, *β*-sitosterol) and at *m/z* 412 (**5**b, stigmasterol), while that compound **6** showed two distinct parent ions at *m/z* 577and at *m/z* 575 with 163 mass unit difference indicating the presence of glucose unit in compound **6** which was confirmed by acid hydrolysis. The previous data beside the significant difference in polarity of compounds **5** and **6** $R_f$ value ($R_f$ 0.8 and 0.68 respectively, solvent system III) meaning that compound **6** is glucosidic derivative of compound **5**. From the previously mentioned data and through direct comparison of (MS, IR, mp and Co TLC) with authentic samples, compound **5** (**5** a and **5** b) and **6** (**6**a and **6**b) were proved to be phytosterol and phytosterol glucoside respectively. According to the available literature, this is the first report about the isolation of compound **6** from *A. lebbeck.*

***Compound 7 stigmasterol glucoside***

By comparing the IR and MS data, TLC and mp of compound **7** with authentic sample, compound **7** was identified as stigmasterol-3-*O*-glucoside.

***Compound 8***

The IR spectrum of compound **8** showed a hydroxyl stretching band at 3420 $cm^{-1}$ and band at 1609 $cm^{-1}$ for carbonyl group. Compound **8** was recognized as flavone compound from its UV absorption maxima at 349 nm (band I) and 254 nm (band II) [32]. The position of the hydroxyl groups on the skeleton was established to be at 5, 7, 3` and 4` positions by the UV shifting reagents. where sodium methoxide showed bathochromic shift (52 nm, band I, OH-4`), aluminum chloride caused a bathochromic shift (72 nm, band I, OH-4`, 3`, 5). Aluminum chloride /HCl caused hypsochromic shift (1 nm) for band I and (20 nm) for band II confirmed the presence of 5, 3` & 4`hydroxy groups. Sodium acetate spectrum exhibited (10 nm) bathochromic shift for band II, indicating the presence of free 7-hydroxyl group. The EI-MS spectrum exhibited a molecular ion at *m/z* 286 [$M^+$] with fragments at *m/z* 258 [$M^+$-CO], 153 [benzoyl moiety of ring **A**] and 135 [for cinnamoyl moiety of ring **B**]. $^1$H NMR spectrum showed the signals of ABX spin system of ring **B** at $\delta_H$ 7.36 (1H, d, *J*=2 Hz), 7.34 (1H, dd, *J*=, 8.6, 2 Hz) and 6.98 (1H, d, *J*=8.6 Hz) for H-2`, H-6` and H-5 respectively. It also showed a singlet signal at $\delta_H$ 6.52 for H-3. The other two protons were at $\delta_H$ 6.42 (1H, d, *J*=1.7 Hz) and 6.18 (1H, d, *J*=1.7 Hz) for H-8 and H-6 respectively. The $^{13}$C NMR spectra showed the carbonyl signal at $\delta_C$ 182.7 (C-4) and C-3 signal at $\delta_C$ 103.1. The oxygen containing carbons showed the downfield signals at $\delta_C$ 165.0 (C-7), 164.7 (C-9), 160.0 (C-2), 158.1 (C-5), 149.6 (C-4`) and 145.7 (C-3`) for oxygen carrying carbons. By comparison of UV, IR, MS and $^1$H & $^{13}$C-NMR of compound **8** with

literature [32] compound **8** could be identified as 3', 4', 5, 7-tetrahydroxyflavone (luteolin). It is isolated for the first time from *A. lebbeck.*

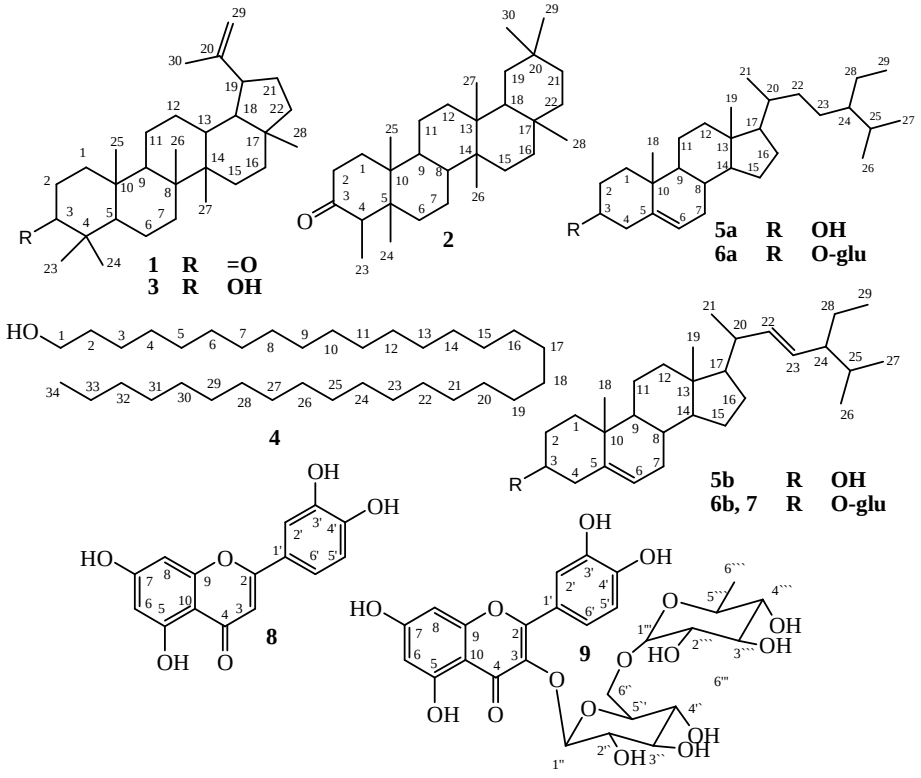


**Figure 3: Structures of compounds 1-9**

***Compound 9***

The UV spectrum of compound **9** showed $\lambda_{max}$ at 358 and 258 nm suggesting flavonol structure [32]. The IR spectrum (KBr) indicated the presence of hydroxyl group as broad band at 3374 $cm^{-1}$, C-O stretching band at 1065 $cm^{-1}$, aromatic =C-H (Peaks at 2925 $cm^{-1}$) and conjugated carbonyl group (absorption band at 1653 $cm^{-1}$). The EI-MS spectrum exhibited the molecular ion of aglycon at *m/z* 302 [$M^+$] with fragments at *m/z* 152 , 150 , 137 , 135 , 108 and 57 which corresponding to the fragmentation pattern of quercetin. The $^1H$ NMR showed the presence of two *meta*-coupled aromatic protons at $\delta_H$ 6.21 (*brs*) and $\delta_H$ 6.41 (*brs*) assigned to H-6 and H-8 respectively. The rest of aromatic protons were assigned for ring-B protons at $\delta_H$ 7.89 (*brs*) assigned to H-2', doublet at $\delta_H$ 7.6 (*d, J*= 8.4) assigned to H-6', and an *ortho*-coupled proton at $\delta_H$ 6.91 (*d, J*= 8.4 Hz) assigned to H-5'. Two anomeric protons signals at $\delta_H$ 5.12 (d, *J*= 7.6 Hz) and $\delta_H$ 4.53 (*brs*) indicating the presence of glucose and rhamnose as sugar moieties respectively. The presence of rhamnose was further confirmed by the signal of terminal methyl appeared as broad singlet at $\delta_H$ 1.30 (3H). The identification of compound **9** was confirmed by Co TLC with authentic rutin. It was separated before from *A. lebbeck* [13].

**Results of analysis of fatty acid methyl ester and unsaponifiable matter of *A. lebbeck***

***1- Results of GLC analysis of fatty acid methyl esters:***

From the results shown in table (1) it could be concluded that thirteen fatty acids methyl esters were detected in *A. lebbeck* stem bark, ten fatty acids were identified and constitute 91.442 %. Six fatty acids (capric, lauric, myristic, palmitic, margaric and arachidic) represent the saturated fatty acids which comprise about 60.17 % of the total analyzed fatty acids. The monounsaturated fatty acid (palmiteolic and oleic) represents 22.365 % of the total fatty acid contents. The diunsaturated fatty acid (linoleic) represents 8.887 % of the total fatty acid contents. Palmitic (33.959 %), Myristic (19.808 %), and Oleic (14.685 %) are the major fatty acids.

***2- Results of GLC / MS analysis of the unsaponifiable matter:***

From table (2), nineteen compounds were identified constituting 55.8% of the total unsaponifiable matter. The identified compounds are: pentadecane (0.53 %), hexadecane (0.84 %), heptadecane (0.73%), 6,10,14-trimethylpentadecanone (4.96 %), nonadecane (0.58 %), eicosane (1.60), phytol (6.53 %), 1-octadecanol(6.19%), 1-cycloeicosane (3.96 %), cyclotetracosane (0.33%), 9-hexacosene (1.33%), cyclooctacosane (0.73%), stigma-5, 22-dien-3-ol (0.51%), *β*- sitosterol – stigmasterol mixture (2.39%), 3-keto-urs-12-ene (1.10%), *β*-amyrene (4.38%), lupenone (10.31%), lup-20 (29)-en-3-ol (7.50%), friedelin (1.57%). Sterols and triterpenes constitute about 24.76 % of unsaponifiable matters where the lupenone is the major constituent.

**Table (1): Results of GLC analysis of fatty acid methyl esters from the light petroleum fraction of the stem bark of *A. lebbeck***

| Retention Time | Area % | No. of Carbon: Double Bond | Systematic Name | Trivial Name |
|---|---|---|---|---|
| 9.033 | 0.17 | 10 : 0 | Decanoic | Capric |
| 10.370 | 2.313 | Unidentified | ---------- | -------- |
| 10.628 | 1.690 | 12 : 0 | Dodecanoic | Lauric |
| 11.482 | 19.808 | 14 : 0 | Tetradecanoic | Myristic |
| 12.335 | 33.959 | 16 : 0 | Hexadecanoic | Palmitic |
| 13.182 | 7.680 | 16 : 1 | *Cis*-9- hexadecanoic | Palmitoleic |
| 13.912 | 1.138 | Unidentified | ---------- | -------- |
| 14.983 | 1.107 | 17 : 0 | Heptadecanoic | Margaric |
| 15.533 | 4.044 | 18 : 0 | Octadecanoic | Stearic |
| 16.05 | 14.685 | 18 : 1 | *Cis*-9-octadecanoic | Oleic |
| 17.017 | 1.081 | Unidentified | ---------- | -------- |
| 17.313 | 8.887 | 18 : 2 | 9,12-Octadecadienoic | Linoleic |
| 20.195 | 1.220 | 20 : 0 | Eicosanoic | Arachidic |

**Results of quantitative estimation of total phenolic and flavonoidal contents of *stem bark extracts of A. lebbeck***

The quantitative estimation of total phenolic and flavonoidal contents of the total alcoholic extract, ethyl acetate and butanol fractions of stem bark of *A. lebbeck* showed that the plant is rich in phenolic and flavonoidal compounds according to the data shown in tables (3 & 4) and the maximum phenolic and flavonoid contents were found to be in the ethyl acetate fraction at concentrations of 104.9007± 0.233 mg GAE/g for phenolics, 165 ± 0.294 mg quercetin equivalent /g and 219.409 ± 0.525 mg rutin equivalent /g extract for flavonoids. It is well known that the plant flavonoids and phenols in general, are highly effective free radical scavenging and antioxidants. Polyphenolics and flavonoids are used for the prevention and cure of various diseases which are mainly associated with free radicals and this interprets the antioxidant effect of ethyl acetate is higher than that of total alcoholic extract.

**Table (2): Results of GLC/MS analysis of the unsaponifiable matter of *A. lebbeck***

| No | Compound Name | Rt | Area % | No | Compound Name | Rt | Area % |
|---|---|---|---|---|---|---|---|
| **1** | Pentadecane | 16.64 | 0.53 | **11** | Cyclotetracosane | 31.66 | 0.33 |
| **2** | Hexadecane | 18.26 | 0.84 | **12** | 9-Hexacosene | 34.34 | 1.33 |
| **3** | Heptadecane | 19.79 | 0.73 | **13** | Cyclooctacosane | 38.14 | 0.73 |
| **4** | Unidentified | 21.19 | 13.85 | **14** | Stigma-5,22-dien-3-ol | 42.79 | 0.51 |
| **5** | 6,10,14trimethyl Pentadecanone | 21.96 | 4.96 | **15** | *β*- sitosterol , stigmasterol mixture | 44.89 | 2.39 |
| **6** | Nonadecane | 22.58 | 0.58 | **16** | 3-Keto-urs-12-ene (α-amirenone) | 45.17 | 1.10 |
| **7** | Eicosane | 22.85 | 1.60 | **17** | *β*- Amyrin | 45.89 | 4.38 |
| **8** | Phytol | 25.44 | 6.53 | **18** | Lupenone | 47.03 | 10.31 |
| **9** | Octadecanol | 27.45 | 6.19 | **19** | Lup-20(29)-en-3ol | 48.09 | 7.50 |
| **10** | 1-Cycloeicosane | 29.60 | 3.69 | **20** | Friedelin | 48.38 | 1.57 |

**Results of qualitative estimation of total phenolic and flavonoidal contents of *stem bark extracts of A. lebbeck***

The results of the HPLC analysis of phenolic contents of total alcoholic extract, ethyl acetate and butanol fractions of *stem bark of A. lebbeck* were recorded in table (5) and revealed the presence of 24 identified compounds representing 15.5 % and 26.0% of the total composition of the total alcoholic extract and the ethyl acetate fraction respectively. e-Vanillic acid represented the major compound in the ethyl acetate and butanol fractions at concentrations of 15079.44 ppm and 2512.53 ppm respectively, while syringic acid represented the major compound in total alcoholic extract at concentration of 3252.61 ppm. In addition, the results of the HPLC analysis of flavonoidal contents of the total alcoholic extract, ethyl acetate and butanol fractions of *A. lebbeck* stem bark were reported in table 6 and showed the presence of 12 identified compounds representing 9.34 %, 25.7 % and 30.5 % of the total composition of the fractions respectively. Luteolin followed by quercetrin and rutin represent the major

compounds found in the ethyl acetate fraction, while hesperidine followed by luteolin are the major compounds in the *n*- butanol fraction. On the other hand, rutin followed by hesperidin are the major compounds in the total alcoholic extract. The compounds were identified by comparing the retention time of their peaks to the retention time of a series of authentics of phenolics and flavonoids injected under the same conditions of the experiment.

**Table (3): Results of total phenolics as gallic acid equivalent of total alcoholic extract, ethyl acetate and butanol fractions of *A. lebbeck* stem bark**

| Plant extract | Absorbance | Total phenolics mg GAE/ g extract |
|---|---|---|
| **Total alcoholic extract** | 317.450 ± 0.005 | 63.490 ± 1.048 |
| **Ethyl acetate fraction** | 524.503 ± 0.006 | 104.901 ± 1.420 |
| **Butanol fraction** | 167.139 ± 0.001 | 33.428 ± 0.232 |

**Table (4): Results of total flavonoids as quercetin and rutin equivalent contents of total alcoholic extract, ethyl acetate and butanol fractions of *A. lebbeck* stem bark**

| Plant extract | Absorbance | Total flavonoids mg quercetin/ g extract | Total flavonoids mg rutin/ g extract |
|---|---|---|---|
| **Total alcoholic** | 0.0015 ± 0.0013 | 27.205 ± 0.588 | 6.681 ± 0.174 |
| **Ethyl acetate fraction** | 0.4695 ± 0.0005 | 165 ± 0.294 | 219.409 ± 0.525 |
| **Butanol fraction** | -0.00175±0.0005 | 26.25 ± 0.416 | 5.205 ± 0.454 |

***Results of biological activities of stem bark extracts of A. lebbeck***

***Anti-inflammatory and analgesic activities***

As shown in table 7 and figure 4 the intradermal injection of 0.1 ml carrageenan (1%) in the rat hind paw significantly increased the paw thickness in all specified time points. On the other hand, oral pretreatment with *A. lebbeck* stem bark extract at a dose of 120 mg/kg significantly decreased rats hind paw edema thickness compared to control group. In addition the results obtained from AUC calculation show that *A. lebbeck* stem bark extract (120 mg/kg) has reasonable anti-inflammatory activity. The results presented in table (8) and figure (5) illustrate the strong analgesic activity of the total alcoholic extract of the stem bark of *A. lebbeck* following their oral injection at a dose level of 120 mg/kg body weight of mice. The extract achieved an obvious pain relieving effect represented in a significant decrease in the total number of writhes produced by acetic acid injection compared to control group. Moreover, the analgesic effect of the extract against acetic acid-induced writhing is more potent than the effect produced by standard. These results open the field for more experiments to provide the extract as a natural and effective alternative to commercial NSAIDs used as analgesics avoiding their side effects. The strong analgesic and moderate anti inflammatory activities of the stem bark of *A. lebbeck* may be due to the presence of steroids and steroidal glycosides such as *β*- sitosterol -stigmasterol mixture, *β*-sitosterol-3-*O*- glucoside and stigmasterol -3-*O*-glucoside [33].

**Table (5): Results of HPLC analysis of phenolic constituents of total alcoholic extract, ethyl acetate and *n*-butanol fractions of *A. lebbeck* stem bark**

| Identified Compounds | Phenolic contents in ppm | | | | | | |
|---|---|---|---|---|---|---|---|
| | Total alcoholic | Ethyl acetate | But. fr. | Identified Compounds | Total alcoholic | Ethyl acetate | But. Fr |
| **Syringic** | 3252.61 | 3806.16 | 388.92 | **Caffeine** | 117.32 | 45.44 | 107.03 |
| **Pyrogallol** | 535.19 | 517.63 | 173.04 | **Ferulic** | 117.13 | 74.54 | 80.96 |
| **Gallic** | 19.41 | 42.22 | 1.95 | **Iso ferulic** | 198.17 | 106.71 | 159.64 |
| **Protocatechuic** | 202.71 | 349.02 | 114.05 | **Benzoic** | 869.36 | 895.26 | 599.05 |
| **Catechol** | 577.46 | 597.15 | 259.82 | **Salicylic** | 1537.77 | 485.9 | 205.89 |
| **4-aminobenzoic** | 9.97 | 28.4 | 11.41 | **Alpha coumaric** | 15.84 | 87.58 | 11.22 |
| **Catechein** | 217.13 | 151.31 | 119.18 | **Coumarin** | 346.55 | 313.86 | 100.68 |
| **Chlorogenic** | 149.22 | 187.27 | 199.14 | **p-coumaric acid** | 519.33 | 158.71 | 119.34 |
| ***P*. OH. Benzoic** | 755.99 | 161.74 | 251.99 | **Ellagic** | 419.77 | 276.4 | 166.44 |
| **Epicatechen** | 218.88 | 260.36 | 456.93 | **3,4,5 methoxy cinnamic** | 43.09 | 25.08 | 42.79 |
| **Caffeic** | 235.49 | 345.4 | 145.97 | **Cinnamic** | 13.66 | 12.91 | 9.44 |
| **Vanillic** | 322.21 | 95.18 | 123.09 | **e-vanillic** | 2455.27 | 15079.44 | 2512.53 |

***Anti-oxidant activity (DPPH free radical scavenging activity)***

As shown in table (9), the total alcoholic extract, ethyl acetate and butanol fractions of *A. lebbeck* stem bark exhibited antioxidant activity. Ethyl acetate fraction showed higher antioxidant activity (55%) than that of total extract (20.2%) and butanol fraction (19.4%) in comparison with ascorbic acid. Antioxidant activity of *A. lebbeck* extract may be due to the presence of flavonoids [17] such as luteolin and rutin.

**Table (6): Results of HPLC analysis of flavonoid constituents of total alcoholic extract, ethyl acetate fraction and *n*-butanol fractions of *A. lebbeck* stem bark in ppm**

| Flavonoidal contents in ppm | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Test items** | **Total alcoholic ex** | **Ethyl acetate** | **But.** | **Test items** | **Total alcoholic ext.** | **Ethyl acetate** | **But.** |
| **Narengin** | 341.9 | 2018.67 | 338.65 | **Quercetin** | 115.35 | 308.62 | 89.12 |
| **Hisperdin** | 591.55 | 2045.58 | 1402.82 | **Hispertin** | 53.96 | 180.78 | 134.79 |
| **Rosmarinic** | 31.95 | 214.38 | 195.72 | **Kampferol** | 157.84 | 908.63 | 128.19 |
| **Rutin** | 604.7 | 3008.37 | 451.71 | **Luteolin** | 399.01 | 6024.92 | 508.36 |
| **Querceterin** | 380.27 | 3120.15 | 445.8 | **Apigenen** | 69.27 | 47.49 | 44.44 |
| **Narenginin** | 50.57 | 115.16 | 47.35 | **7-hydroxyflavone** | 48.77 | 32.42 | 57.17 |

***Antimicrobial activity***

The results of preliminary antimicrobial activity table 10 revealed that all the tested extracts have a moderate antibacterial effect against the tested Gram -ve bacteria except butanol extract of the stem bark which has mild antibacterial activity against *Pseudomonas aeruginosae* ATCC 27853. For the tested Gram -ve bacteria all the extracts have shown a moderate antibacterial effect relative to the standard amoxycillin. The extracts have shown moderate antifungal effect against *Aspragillus niger* and *Candida albicans* relative to the standard amphotericin B. These results indicate the potential use of *A. lebbeck* in management of bacterial diseases caused by *Escherichia coli*, *Pseudomonas aeruginosa* , *Staph. aureus* and *Staph. epidermis* since these bacteria's are an important pathogenic bacteria causing a large number of diseases in human being and animals. The antimicrobial effect may be due to the presence of sterols and sterol glucoside [34].

***Cytotoxic activity***

The cytotoxic activity was carried out according to the method reported by Mosmann 1983 [35]. The results of cytotoxic activity of stem bark methanolic extract of *A. lebbeck* against HePG-2 (Hepatocarcinoma), HCT-11 6 (Colon carcinoma), HEP-2 (Larynx carcinoma), HELA (Cervical carcinoma) and MCF-7 (Breast carcinoma) cell lines were reported in table 11 and it showed that the extract exhibited strong cytotoxic activity against all tested cell lines with $LD_{50}$ 5.2, 11.1, 11.7, 44.0 and 48.9µg respectively.

**Table (7): Effect of total alcoholic *A. lebbeck* extract (120 mg/kg.) on hind paw thickness at different time intervals after induction of oedema using carrageenan**

| Treatment | Percentage increase in oedema thickness | | | | | | Total % decrease in oedema thickness | |
|---|---|---|---|---|---|---|---|---|
| | Time hrs | | | | | | | |
| | **1** | **2** | **3** | **4** | **5** | **24** | **AUC** | **Total % inhibition** |
| **Control** | 27.39±3.67 | 34.99±4.37 | 42.08±4.14 | 52.29±5.73 | 42.02±5.13 | 13.54±3.42 | 705.5 | 0% |
| **Diclofenac sodium** | 32.93±2.28 | 39.10±1.20 | 29.87±2.7 | 30.71±3.00* | 18.79±5.34 | 6.78±3.14* | 385.0 | 45.43% |
| **EtOH extract** | 34.73±7.20 | 46.69±6.19 | 32.83±7.32 | 33.37±4.22* | 29.36±5.43* | 2.56±2.14* | 465.2 | 34.06% |

*AUC= area under curve * Significant different from control group.*

**Table (8): Results of analgesic activity the total alcoholic extract of *A. lebbeck* stem bark administered orally at a dose of 120 mg/kg in acetic acid-induced writhing model**

| | **Control** | ***A. Lebbeck* extract** | **Diclofenac sod.** |
|---|---|---|---|
| **Total number of writhes** | 61.33 ± 5.24 | 5.5 ± 5.17* | 23 ± 5.03* |
| **% inhibition** | 0% | 91% | 62% |

** Significantly different from control group.*

**Table (9): Antioxidant activity of total alcoholic extract, ethyl acetate and butanol fraction of *A. lebbeck* stem bark**

| **Extract** | **Absorbance ($A_1$)** | **A.-A1** | **Antioxidant activity %** |
|---|---|---|---|
| **Total alcoholic extract** | 0.202 | 0.798 | 20.2% |
| **Ethyl acetate fraction** | 0.550 | 0.45 | 55.0% |
| **Butanol fraction** | 0.194 | 0.806 | 19.4% |
| **Ascorbic acid** | 0.969 | 0.031 | 96.9% |

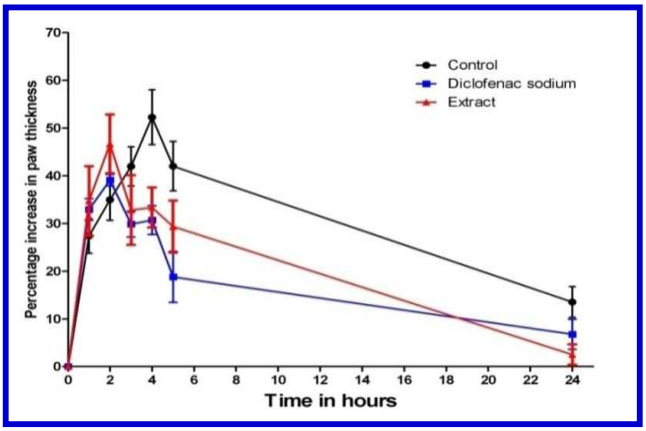


**Figure 4: Effect of *A. lebbeck* extract (120 mg/kg.) on hind paw thickness at different time intervals**

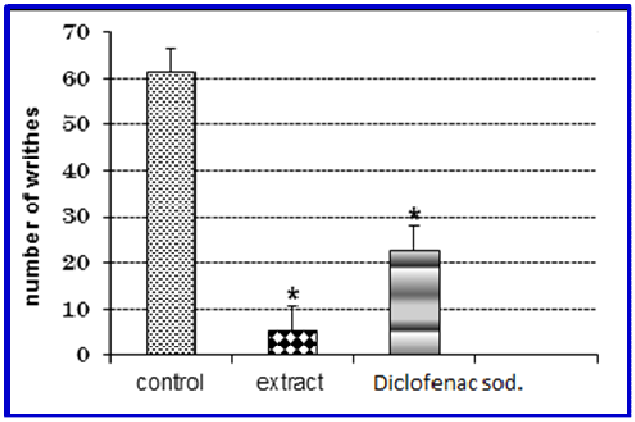


**Figure 5: Evaluation of the analgesic activity of the total alcoholic extract of the stem bark of *A. lebbeck* (120 mg/kg, orally) on acetic acid- induced model in mice**

**Table (10): Results of antimicrobial screening of different extracts of *A. lebbeck* stem bark extracts and amoxicillin and amphotricin B as positive standards**

| Material | Inhibition zone diameter (mm/mg sample) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bacteria | | | | | | Fungi | |
| | Gram –ve | | | Gram +ve | | | | |
| | *E. coli* ATCC 25922 | *E. coli* ATCC 10536 | *Pseud. Aeruginosae* ATCC 27853 | *Staph aureus* ATCC 25923 | *Staph aureus* ATCC 6538 | *Staph Epidermis* ATCC 12228 | *Candida albican* ATCC 10231 | *Aspergillus niger* ATCC 16404 |
| **Amoxycillin** | 25 | 26 | 23 | 35 | 34 | 29 | - | - |
| **Amphotericin B** | - | - | - | - | - | - | 20 | 20 |
| **EtOH extract** | 18 | 18 | 18 | 19 | 19 | 19 | 18 | 19 |
| **Light pet. fraction** | 17 | 16 | 15 | 13 | 17 | 15 | 16 | 15 |
| **Cloroform fraction** | 16 | 15 | 15 | 17 | 17 | 17 | 16 | 16 |
| **Butanol fraction** | 17 | 16 | 12 | 15 | 15 | 16 | 14 | 15 |

*Conc. of standards = 5 mg Conc. Of extract = 100mg*

**Table 11: Results of cytotoxic activity and $LD_{50}$ in µg of stem bark methanolic extract of *A. lebbeck* against different cell lines**

| Sample conc. (in µg) | % Viability for extract against different cell lines | | | | |
|---|---|---|---|---|---|
| | MCF-7 | HepG-2 cell | HCT-116 | HEP-2 | HELA cell |
| **50** | 10.12 | 17.84 | 21.75 | 43.26 | 48.60 |
| **25** | 18.34 | 30.98 | 34.69 | 71.52 | 79.15 |
| **12.5** | 29.76 | 43.29 | 45.63 | 88.09 | 90.62 |
| **6.25** | 41.47 | 72.31 | 78.57 | 94.18 | 96.97 |
| **3.125** | 66.92 | 86.29 | 91.84 | 98.74 | 100 |
| **1.56** | 83.08 | 93.14 | 97.26 | 100 | 100 |
| **0** | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| **$LD_{50}$ in µg** | 5.2 | 11.7 | 11.1 | 44.0 | 48.9 |

**Acknowledgement**

The authors would like to acknowledge and thank Nora Hassan Assistant Lecturer of Pharmacology, Pharmacology Department, Faculty of Pharmacy, Zagazig University for carrying out the pharmacological screening. Our thanks due to Nader Shawky Assistant Lecturer of Microbiology, Microbiology Department, Faculty of Pharmacy, Zagazig University for carrying out the antimicrobial activities.

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