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Research Article

Antiviral, Cytotoxicity, Antioxidant and Chemical Constituents of *Adansonia digitata* Grown in Egypt

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ABSTRACT

The Areal parts of Adansonia digitata were successively extracted by gradient solvents. The resulting extracts were submitted to bioassay in order to uncover their biological potential. The antiviral activity indicated significant activities: adenovirus type-7 56.6%(MeOH extract) and 66.6%(water extract) while rotavirus Wa strain 63.3% and 70% and coxsackievirus B4 70% titre reduction, while the HepG2 cytotoxicity (IC_{50} 19.3 µg/ml vs 24.46 µg / ml for doxorubicin), analgesic (82.5% vs 79.6% for standard aspirin), antioxidant (38.3 µg/ml vs 4.80 µg/ml for vitamin C) as well as the anti-inflammatory (61.5% vs 59.5% for indomethacin) indicated promising activities. Repeated chromatographic columns for these extracts afforded nine chemical compounds. Spectral analysis of these compounds using UV, MS and NMR confirmed the presence of several flavonoids: apigenin, luteolin, Genkwanin, Luteolin -7-*O*- galactoside, Chrysoeriol-7-*O* - glucoside and Luteolin -7-*O*- glucoside. In addition, α-amyrin palmitate, Ursolic acid and Ursolic acid derivative [24-(Trans) Ferulyl) Oxy-3-Hydroxy-Urs-12-En-28-Oic acid] were obtained for the first time from the plant. Total plant flavonoids (3.73 mg/100 mg leaves extract and 1.491 mg /100 mg fruit pulp) and Vit C content (9.33 mg/100g leaves extract and 0.8 mg/100g seed extract) were also determined.

Keywords: *Adansonia digitata*, antiviral, cytotoxicity, antioxidant, flavonoids, triterpenes.

INTRODUCTION

The plant family Bombacaceae contains about 250 species in 30 genera, widely distributed in tropical regions all over the world. The genus Adansonia comprises ten species of tropical shrubs and trees. In Egypt, only one species (Adansonia digitata =Adansonia bahobab) is growing in upper Egypt as large woody tree considered as one of the most important food plants, providing fruits, berries, nuts, seeds and leaves and the same species in South Africa[1,2]. Obviously, no literature records concerning the Egyptian tree either phytochemically nor biologically, while the southern Africa one is reported to be used in an exceedingly wide range of medicinal and traditional uses with numerous applications in several folkloric disorders[3,4]. Also, this species reported to possess an antioxidant[5] and antiviral activities[6-11]. Therefore, it was part of our research program to undertake the present study of this Egyptian species to uncover its pharmacological potential and chemical constituents.

MATERIALS AND METHODS

Plant material

Adansonia digitata L. leaves and fruits (ripe and unripe) were collected from Plant Island in Aswan, Egypt around September 2009. The plant was kindly authenticated by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre and Dr. Tereez Labib Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen was kept in the Herbarium of the National Research Centre, Cairo, Egypt (Reg. no. M 99). The leaves were air dried in the shade and reduced to powder. Fruits were opened; fruit pulp and seeds were separated manually.

Chemicals

Indomethacin capsules (20mg/kg) was purchased from Kahira Pharmaceutical and Chemical Company, Cairo, Egypt. Carrageenan was purchased from Sigma –Aldrich Chemical Company (USA). Acetyl salicylic acid (Aspirin) was obtained from BDH Chemicals, England. Ascorbic acid and 1,1-diphenyl-2-picryl-hydrazil (DPPH) were purchased from Sigma® Chemical Company (EUA). Adrinamycin (Doxorubicin) was purchased from Sigma – Aldrich Chemical Company (USA). All others chemicals, solvents and reagents used in the chromatography were of analytical grade.

Antiviral activity

Hep-2, MA104 and BGM cell lines were obtained from the Holding Company for Biological Products & Vaccines VACSERA, Egypt). The different leaves and fruit extracts were examined for its antiviral activities against three different viruses, Rotavirus; Human adenoviruses (Ads).

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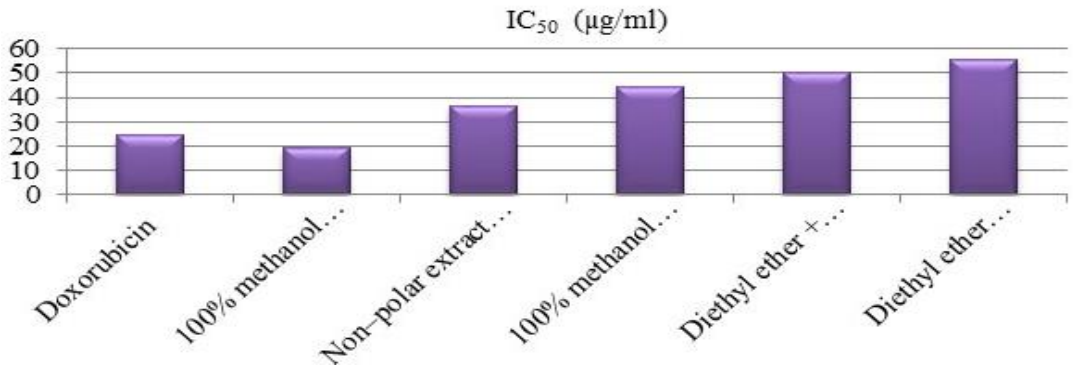


Figure 1: Cytotoxic activity of Adansonia digitata extracts.

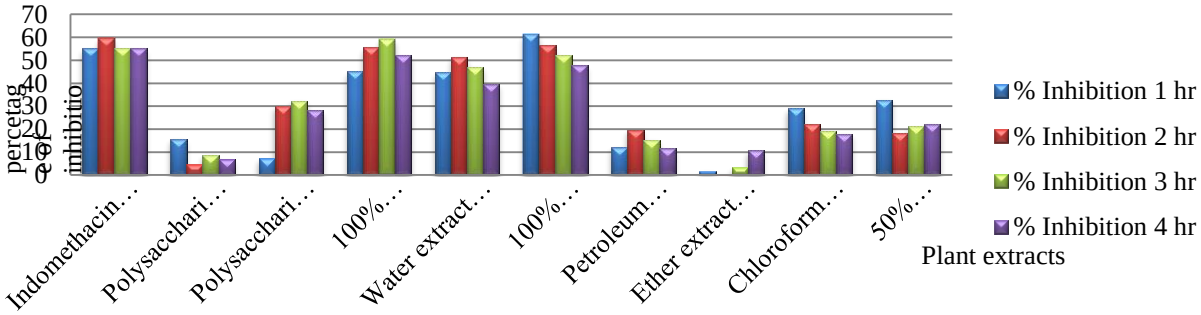


Figure 2: Anti-inflammatory activity of *Adansonia digitata* extracts

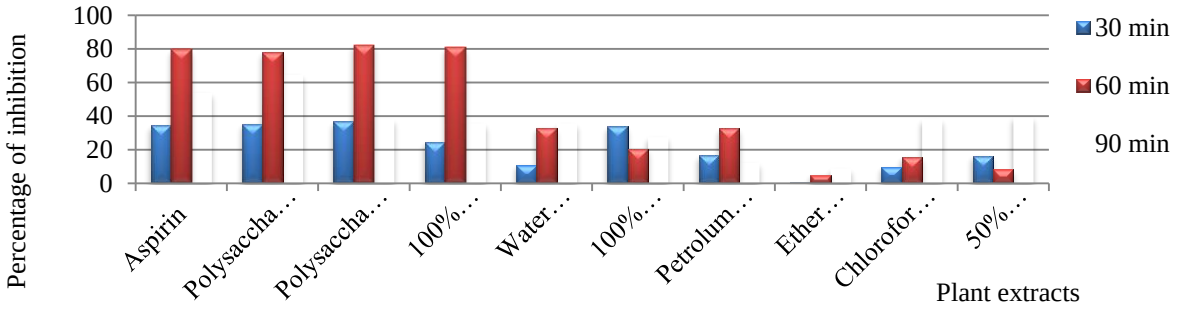


Figure 3: Analgesic activity of Adansonia digitata extract.

The activity was conducted according to the previously reported methods[9-11] for Cell viability assay. The determination of adenovirus type- 7, rotavirus Wa strain, and Coxsackievirus B4 titers using plaque assay according to a previously reported method[11].

Cytotoxic activity

HepG2 (human Liver carcinoma) was obtained from Karolinska Institutet, Stockholm, Sweden. The cells were maintained in RPMI 1640 (Lonza Biowahittkar) medium), and media were supplemented with 1% antibiotic-antimycotic mixture. The cell viability was investigated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[12].This reaction depends on the mitochondrial dependant reduction of yellow MTT into purple formazan. All the preceding steps were carried out in sterile laminar flow cabinet biosafty class II level. The percentage of change in viability was calculated according to the formula:

(Absorbance of tested extract/ Absorbance of negative control) -1) x 100

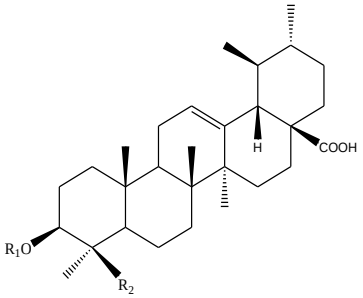
The results of bioassay were analysed according to reported methods[13]. A statistical significance of the cytotoxic assay was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. A probate analysis was carried for IC_{50} determination using SPSS 11 program, Fig 1.

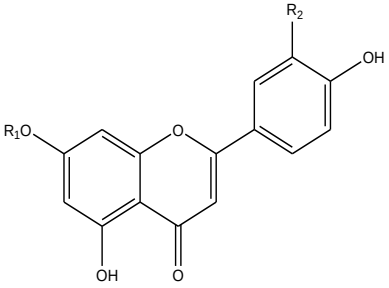
Anti-inflammatory activity

The anti-inflammatory activity of the plant extracts was determined using the carrageenan-induced rat paw oedema model[14,15]. The Animals used were adult white albino rats (n=66) of both sexes weighing 150-200g were obtained from animal house (NRC) and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication No. 85-23, revised 1985), Fig 2.

Analgesic activity

This test was carried out using the hot-plate method[16], performed on rats by using an electronically controlled hot-plate to evaluate the potential centrally-mediated analgesic effect of the different extracts. All plant extracts





were injected orally 30 minutes before placing the animal on the hot plate, the results were recorded, Fig 3.

Anti-oxidant activity

The free radical scavenging activity of extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using a reported method[17,18]. Eleven plant extracts were screened at 100 µg/ ml while the most potent active extracts (gave more 90%) were assayed at 25-75µg/ ml. The inhibition % of DPPH radical scaverning activity was calculated according to the following formula: Inhibition (%) = 100 − [$((A_0-A_1)/A_0) \times 100$]

Where: A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample (extract/standard ascorbic acid) The amount of samples necessary to decrease the absorbance of DPPH (IC_{50}) by 50% was calculated.

Extraction and Isolation:

Powder of leaves and fruit pulb were successively extracted with: petroleum ether, diethyl ether, chloroform, ethyl acetate and methanol. The ethyl acetate (3.1 g) was chromatographed on silica column (45g, 15×3 cm) and eluted with chloroform followed by chloroform / ethyl acetate mixtures of increasing polarities affording six fractions (I-VI) fractions III and V were the major, on crystallization (80% ethyl acetate or methanol) afforded compounds 1-9:

Compound 1: yellowish powder (10mg), mp. 345-347ºC, R_f 0.77($CHCl_3$: MeOH 9:1), UV (MeOH) λmax, nm: 268,297 (sh),334; EIMS: m/z 270 (M^+), 152, 118; ^{1}HNMR(CD3OD): δ 7.8 (2H, d, *J* =8.4 Hz, H -2', H 6'), 6.9 (2H, d, *J* =8.4 Hz, H-3', H-5'), 6.6(1H, s, H-3), 6.4 (1H, s, H-8), 6.2 (1H, s, H-6).

Compound 2: obtained as yellow powder (12mg), mp 328-330ºC, Rf 0.49 (BAW 4:1:5); UV (MeOH) λmax, nm: 253, 269(sh), 294 (sh),348; MS(m/z)286 (M^+), 258,152,134; ^{1}HNMR (600 MHz, CD3OD): δ 7.43 (1H, d, *J* =2.2 Hz, H-2'), 7.41 (1H, dd, *J* =2.5 &8.5 Hz, H- 6'), 6.9(1H, d, *J*=8.5 Hz, H -5'), 6.8 (1H, d, *J* = 2.5 Hz, H-8), 6.6 (1H, s, H -3), 6.5 (1H, d, *J* =2.5 Hz, H -6).

Compound 3: yellow powder (15 mg), mp 277-279°C, Rf 0.33(CHCl3: MeOH 8.5:1.5), UV (MeOH) λmax, nm: 262, 340; EIMS:m/z 284(M^+) 152, 127; ^{1}HNMR (600 MHz, CD3OD): δ 7.43 (1H, d, *J* =2.2 Hz, H- 2'), 7.41 (1H, dd, *J* =2.2 &8.5 Hz, H- 6'), 6.9(1H, d, *J*=8.5 Hz, H -5'), 6.8 (1H, d, *J* = 2.5 Hz, H-8), 6.6 (1H, s, H -3), 6.5 (1H, d, *J* =2.5 Hz, H -6), 3.85 (1H, s, OCH3-C7).

Compound 4: colorless oil (18 mg), Rf 0.35(CHCL3: EtOAc 1:1), gives violet color with vanillin-sulphuric reagent and no color with $FeCI_3$; EIMS:(m/z) 664 (M^+), 420, 394, 376, 276, 248, 218, 203, 189,131,108, 81, 69 and 56; ^{1}H NMR (CDCl3 ,600 MHz), δ 5.2 (H-12), 4.3 (H-3 α), 2.29 (H-2) ,2.10 (H-18), 1.18 (-CH2 fatty acid chain), 1.07- 0.77 (eight methyls); ^{13}CNMR (CDCl3 ,75 MHz): δ 79 (C-3), 124. (C-12), 140 (C-13), 173 (O-CO-C-3α), 29.7 (CH2-fatty acid chain), 14.3 (CH_3- fatty acid chain).

Compound 5: white crystals (18mg), mp 280-282°C, Rf 0.45(CHCl3: MeOH 9:1), It gave positive Lieberman-Burchard and Salkowiski's tests; EIMS (m/z) 456 (M^+), 410, 248, 207,203, 189, 133,81, 69, 55.

*C*ompound 6: amorphous powder (25mg), mp 200-202ºC, Rf 0.16($CHCl_3$: EtOAc 9:1); EIMS (m/z): 648 (M^+) (1.74) ,630 (7.08), 602(11.33), 453 (2.07), 407 (1.34), 248 (2.87), 236 (13.84), 207 (3.70) ,203 (7.55) ,189 (2.58), 133 (2.36) ,81 (16.34), 78(100%).

Compound 7: yellow powder (20 mg), mp 255ºC, Rf 0.35(Acetic acid: H2O, 1:1); UV (MeOH) λmax, nm: 254, 268(sh.), 348; EIMS (m/z): 286(M^+), 258, 153; ^{1}HNMR (DMSO-*d6*, 600 MHz): δ 7.418 (1H, m, 2'/6'-H), 6.91 (1H, d, *J*=8.5,5'-H), 6.75, 6.39 (1H, d, 6 /8-H *J* = 2), 6.71 (1H, s ,3-H), 5.04 (1H, d, H-1`` *J*= 6.7) and 3.9 -3.4 (H -2" - H-6").

Compound 8: yellow powder (20 mg), mp 174 -176ºC, Rf 0.33 (Acetic acid: H2O 1:1), UV(MeOH) λmax, nm: 253, 268(sh.), 348; EIMS(m/z):300 (M^+), 286, 259,213,152 ^{1}HNMR (DMSO-d6, 600MHz): δ 7.453 (1H, *dd*, *J* =8.5, 2 Hz, H-6'), 7.41 (1H, *d*, *J*=1.9 Hz, H-2'), 6.91 (1H, *d*, *J*= 8.1 Hz, H-5'),6.73 (1H, *s*, H-3), 6.78 (1H, *d*, *J*= 2 Hz, H-8), 6.44(1H, *d*, *J*= 2 Hz, H-6), 3.87 (3H, *s*, OMe), sugar moiety: δ 5.05 (1H, *d*, *J*= 6.9 Hz, H-1").

Compound 9: yellow powder (32 mg), mp 260ºC; Rf 0.34(Acetic acid: H2O 1:1), UV (MeOH)λmax, nm: 253, 268(sh.), 347; EIMS(m/z) :286 (M^+), 258,229,153, ^{1}HNMR (DMSO-d6, 600MHz): δ7.408 (1H, m, 2'/6'-H), 6.88 (1H, d, 5'-H, J=8.5), 6.58 (1H, s ,3-H), 6.77, 6.47 (1H, d, 6 /8-H), 5.04 (1H, d, H-1``, J= 7.5).

Acid hydrolysis

Each compound (5.0 mg) was refluxed with 1.5 N H2SO4 in aqueous 50% methanol for 2 hours. The reaction was completed according to reported method**[19].** The resulting sugars and aglycones were co-chromatographed against authentic sugars and flavonoids.

Determination of total flavonoid content

The total flavonoidal content was determined according to previous colorimetric method[20] and a standard curve of rutin as reference. The absorbance of the color developed was measured at λmax 415 nm after 30 min against a blank sample (consisting of 3ml AlCl3 and 3 ml methanol).

Quantitative estimation of vitamin C

S. No	Compound	R_1	R_2
1.	Apigenin	H	H
2.	Luteolin	H	OH
3.	Genkwanin	CH_3	H
4.	α-amyrin-3-palmitate	Palmityl	CH_3
5.	Ursolic acid	H	CH_3
6.	24-(trans-ferulyl) oxy-3-hydroxy-urs-12-en-28-oic acid	H	Ferulyl
7.	Luteolin-7-O-galactoside	β-D-galactose	OH
8.	Chrysoeriol-7-O- β-D-glucose	β-D-glucose	OCH_3
9.	Luteolin-7-O-glucoside	β-D-glucose	OH

This determination was carried out on the dried leaves, fruit pulp and seeds by using the internationally reported method[21].

RESULTS AND DISCUSSION

A. digitata is the only Egyptian species of the genus Adansonia. The areal parts were extracted by gradient solvents and the resulting extracts were submitted to intensive biological and chemical investigations.

Biological Study

Antiviral activity

The test results for this activity[10,11,22-25] indicated slight increasing of resistance to toxic materials in BGM cell line more than Hep-2 and MA104 cell lines. Both methanol extract and water extract of leaves revealed significant reduction in the titre of adenovirus type-7 56.6% and 66.6% respectively, rotavirus Wa strain 63.3% and 70%, respectively and Coxsackievirus B4 70% for both of them. On the other hand, polysaccharide of pulp revealed significant reduction 50% of the titre of Coxsackie virus B4 while it did not give significant decrease in the titre of both rotavirus Wa strain and adenovirus type-7 (< 50%).

Cytotoxic activity

Eight extracts from the leaves: petroleum ether, diethyl ether, chloroform, pet. ether+diethyl ether, diethyl ether + chloroform, pet. ether + chloroform, 50% methanol extract,100% methanol extract, water extract, were tested for *in vitro* cytotoxicity. The most effective and significant[12,13] extract was 100% methanol with (IC_{50} = 19.3 µg/ml) against the reference drug (doxorubicin IC_{50} = 24.46 µg / ml)), Fig 1.

Anti-inflammatory activity

In this study, Indomethacin had its higher effect in the second hour (59.5%) while 100% methanol extract of leaves produced a significant (P< 0.05) inhibition of the edema after the first hour (61.5%) according to previous method[15,16], Fig 2.

Analgesic Activity

Significant analgesic activity (82.5%) was obtained in comparison with standard aspirin (79.6%) after 60 min according to previously reported method[16], Fig 3.

Antioxidant activity

The free radical scavenging activity of extracts was measured by DPPH method[17,18]. The most potent active extracts (gave 90%); were (100%methanol extract of leaves and 100% methanol extract of pulp) against standard vit C (IC_{50} = 4.8 µg/ml).

Chemical study

The methanolic extract of leaves and fruit pulp of A. digitata, was investigated on several chromatographic columns and resulted in isolation of nine compounds. Their structures were established using different spectral techniques uv, ms and ^{1}Hnmr:

Compound 1 yellowish-white powder, exhibited uv and shift reagents suggesting the flavonoidal nature[26]. The MS (M^+ 270) and other fragments confirmed the presence of 5,7,3' trihydroxy flavone. Collectively, these data confirmed the structure to be apigenin[27].

compound 2 yellow powder, uv and shift reagents as well as MS with parent ion M^+ 286 and fragments 153, 134 suggested the presence of tetrahydroxy flavonoid. Comparison with published data[12] confirmed the structure to be luteolin.

compound 3 yellow powder showed uv with shift reagents (occupied C-7 OH), MS (M^+ 284, 253, 167) and ^{1}Hnmr with a singlet at δ 3.85, as well as comparison with published data for 4',5-Dihydroxy-7-methoxy flavone, genkwanin[26].

compound 4 colorless oil; ms with M^+ m/z 664 and fragments d at 420, 276, 248, 232, 218, 203 ,189, 131, 108, 81, 69, 56 suggesting the presence of triterpenoidal structure[28]. The ^{1}H-NMR showed δ4.5 (1H, *m*, H-3 α), 1.18 (28H, brs, -CH_2 fatty acid chain), 1.07-0.77 (3H, brs, for 8 methyls). These data were co-incident with those published for α-amyrin palmitate[29,30]. This is the first report on isolation of this compound from the Egyptian plant.

Compound 5 obtained as white solid; ms M^+456 (410, 248, 207,203, 189,133 typical of ursane skeleton[28]. The obtained data were identical with those published for ursolic acid[31].

Compound 6 white solid; ms with parent peak at 456 (M^+), 248 and 207significant of Δ^{12}-unsaturated triterpene. ^{1}Hnmr signal at δ 5.2 and ^{13}CNMR signal at δ 125.4, 137.7for C-12 and C-13, doublet at δ2.17 (J= 11 Hz) for the allylic 18β-H significant for compounds with Δ^{12}-ursene derivative substituted in D and E rings with a free COOH group at C-28 (δ_C183.5, m/z 410) confirmed the presence of ursolic moiety. ^{13}Cnmr at δ 22.2 and 65.5 for C-23 and C-24 with the down-field of C-24 indicating esterification at the hydroxymethyl group with trans-ferulic acid. From the above mentioned data and comparison with published reports[32,7] this compound was identified as [24-(trans) ferulyl) oxy-3β-hydroxy-urs-12-en-28-oic acid]). This is the first report on isolation of this compound from the Egyptian plant and second to the Senegal species[7].

Compound 7 yellow powder, uv with shift reagents showed flavonoidal glycoside skeleton; ms (M^+) 286; ^{1}Hnmr and acid hydrolysis confirmed the structure of the compound to be Luteolin -7-*O-D*-galactoside. It is the first report on this isolation from the genus.

Compound 8 yellow powder; its uv with shift reagents; ms (M^+ 300) and significant fragments 286, 258,152,134 suggesting the structure to be tetrahydroxy flavone substituted at C-7 and C-3'. ^{1}Hnmr exhibited a singlet at δ 3.87 for methoxyl at C-3'. Acid hydrolysis afforded chrysoeriol and glucose. The structure was found identical with reported data[33]. This is the first report on isolation of this compound from the genus.

Compound 9 yellow powder; uv and ms (M^+ 286) proved the structure to be tetrahydroxy flavone substituted at C-7. Acid hydrolysis afforded luteolin and glucose. This is the first report on the presence of luteolin 7-O- glucoside in the plant genus.

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REFERENCES

1. Bailey, LH. The Standard Cyclopedia of Horticulture, Vol. 1. The Macmillan Company, New York, 1947, 214-215.
2. Palmer E and Pitman N. Trees of Southern Africa. Vol. 2, Balkema, Cape town, 1972,2.
3. Vimalanathan S and Hudson J. Multiple inflammatory and antiviral activities in Adansonia digitata (baobab) leaves, fruits and seeds, J. Med. Plants Res., 2009, 3(8): 576-582.
4. Ramadan A, Harraz F and El-Mougy S. Anti-inflammatory, analgesic and antipyretic effects of the fruit pulp of Adansonia digitata, Fitoterapia, 1994,65(5): 418-422.
5. Compaoré W R, Nikièma PA, Bassolé HIN, Savadogo A, Mouecoucou J, Hounhouigan DJ and Traoré SA, Chemical Composition and Antioxidative Properties of Seeds of Moringa oleifera and Pulps of Parkia biglobosa and Adansonia digitata Commonly used in Food Fortification in Burkina Faso, Current Res.J. Biological Sciences, 2011, 3(1), 64-72.
6. Hudson JB, Anani K, Lee MX, De Souzâ C, Arnason JT and Gbeassor M. Further investigations of the antiviral activities of medicinal plants of Togo, Pharmaceutical Biology, 2000, 38(1): 46-50**.**
7. Al-Qarawi AA, Al-Damegh MA and El-Mougy SA, Hepatoprotective influence of Adansonia digitata pulp, J. Herbs, Spices & Medicinal plants, 2003,10 (3): 2-6, C A: 10 (2003).
8. Atmar RL, Openkun AR, Gilger MA, Estes MK, Crawford SE, Neill FH, and Graham DY, Norwalk viruses shedding after experimental human infection. Emerging Infectious Diseases, 2008, 14: 1553-1557.
9. Hamdy NA and El-Senousy WM. Synthesis and antiviral evaluation of some novel pyrazoles and pyrazoles and pyrazolo[3,4-d] pyridazines bearing 5,6,7,8-tetrahydro-naphthalene. Acta Poloniae Pharmaceutica journal- Drug Research, 2011, 70(1): 99-110.
10. Hamdy NA, El-Senousy WM, and Fakhr IMI. Enaminone as building blocks in organic chemistry: a novel route to polyfunctionally 2- substituted 5, 6, 7, 8-tetrahydronaphthalenes and their antiviral evaluation. J. Heterocyclic Chemistry, 2013, 50: 337-343.
11. Salem M, Sakr S, El-Senousy WM and Madkour H. Synthesis, antibacterial, and antiviral evaluation of new heterocycles containing the pyridine moiety. Archiv der Pharmazie, 2013, 346 (10), 766-773.
12. Mosmann T**.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J Immunol. Meth., 1983, 65: 55-63.
13. Armitage P**.** Statistical methods in medical assays. 1st. ed., Black-well Scientific Publications, London, 1971, 147.
14. Chattopadhyay D, Arunachalam G, Mandal AB, Sur TK, Mandal SC and Bhattacharya SK, Antimicrobial and antiinflammatory activity of folklore: Mallotus peltatus leaf extract. J. Ethnopharmacol. 2002, 82: 229-237.
15. Woolfe G and McDonald A. The evaluation of the analgesic action of pethidinehydrochloride (DEMEROL), J. Pharmacol. Exper. Ther.,1944, 80: 300-307
16. Oktay M, Gülçin I, Küfrevioˇlu I. Determination of *in-vitro* antioxidant activity of fennel (Foeniculum vulgare) seed extracts, Lebensmittel-Wissenchaft and Technologie, 2003, 36: 263-271.
17. Ratty AK, Sunamoto, NP Das. Interaction of flavonoids with 1, 1- diphenyl – 2- picrylhydrazyl free radical, liposomal membranes and soybean lipoxygenase-1. Biochem Pharmacol. 1988, 37(6):989-95.
18. Harborne JB, Mabry TJ, Mabry H. The Flavonoids.Chapman and Hall Ltd, London, 1975.
19. Meda A, Lamien CE, Romito MM, Nacoulma OG. Determination of total phenolic, flavonoid and proline content in Burkina Fasan honey, as well as their radical scavernging activity. Food Chemistry, 2005, 91(3): 571-577.
20. AOAC. Official Methods of Analysis of the Association of Official Analytical Chemist. 13th Edition (ed. Horwitz, W.) Published by AOAC, POB 540 Benjamin Franklin station, Washington DC, 2004, 746.
21. Bassyouni FA, Saleh TS, Hefnawi El M, Abd El-Moez SI, El-Senousy WM *et al*. Synthesis, pharmacological activity, evaluation and molecular modeling of new polynuclear heterocyclic compounds containing benzimidazole derivatives, Archive of Pharmacal Research ARPR, 2012; 35:2063-2075.
22. Cavallier – Gratacap B, Genoulaz O, Brengel-Pesce1 K, Soule H, Innocenti-Francillard P, Bost M *et al.* Detection of Human and Animal Rotavirus Sequences in Drinking Water. Appl. Enviro. Microbiology. 2000,66(6): 2690-2692.

23. Costafreda MI, Bosch A, Pinto RM. Development, evaluation and standardization of a real-time Taq Man reverse transcription-PCR assay for quantification of hepatitis a virus in clinical and shell fish samples. Appl. Enviro. Microbiology, 2006, 72: 3846-3855.
24. Schmidtke M, Knorre C, Blei L, Stelzner A, Birch-Hirschfeld E. Penetration and antiviral activity of coxsackievirus b3 (cvb3) – specific phosphorothioate oligodeoxynucleotides (ps-odn). Nucleosides and Nucleotides, 1998, 17: 1557-1566.
25. Markham JR. Techniques of Flavonoids Identification, Academic Press, London, 1982.
26. Fawkeya A, Abdel-Monem Ateya. Estradiol, Esteriol, Estrone and Novel Flavonoids from Date Palm Pollen, Aust. J. Basic and Applied Sciences, 2011, 5(8): 606-614.
27. Budzikiewicz H, Djerassi C, Williams DH. Structure Elucidation of Natural Products by Mass Spectroscopy, Holden-Day INC, London, 1964, 2.
28. Serreqi AN, Leone R, Del Rio LF, Mei Sh, Fernandez M, Breuil C. Identification and Quantification of Important Steryl Esters in Aspen Wood, JAOCS, and Journal of the American Oil Chemists' Society, 2000, 77(4): 413-418.
29. Mallavadhani UV, Mahapatra A, Jamil K, Reddy PS. Antimicrobial Activity of Some Pentacyclic Triterpenes And Their Synthesized 3-*O*-Lipophilic Chains, Biol. Pharm. Bull. 2004; 27(10): 1576-1579.
30. Takahashi K, Kawaguchi S, Nishimura K, Kubota K, Tanabe Y, Takani M. Studies on constituents of medicinal plants. XIII. Constituents of the pericarps of the capules of Euscaphis japonica Pax. Chem. Pharm. Bull, 1974, 22: 650.
31. Duh CY, Pezzuto JM, Kinghorn AD, Leung SL, Farnsworth NR. Plant Anticancer Agents XLIV. Cytotoxic Constituents from Stizophyllum reparium, J. Natural Product (Lloydia) 1987, 50: 63-74.
32. Sarkhail P, Esfehani HRM, Amin G, Surmaghi MHS, Shafiee A. Phytochemical study of Phlomisolivieribenth. And Phlomis persica boiss, DARU- J. Pharm. Sci., 2006,14(3): 115-121.