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RESEARCH ARTICLE

Isolation and Identification of Flavonoids from Heartwood of *Acacia nilotica* var *nilotica* and their Antibacterial Activity.

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Abstract

From the methanolic extract of the heartwood of *Acacia nilotica* var *nilotica*, two compounds (I and II) were isolated; compound I: 7, 3', 4'-trihydroxy-3-methoxyflavone and compound II: 7, 3', 4'-trihydroxyflavonol. The compounds were purified by different chromatographic techniques and identified via spectroscopic tools: IR, UV, ¹HNMR and Mass spectroscopy. The isolated compounds were evaluated for their antimicrobial potential against Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram positive (*Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*) bacteria. Both compounds showed varying antibacterial activity.

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Introduction:-

Flavonoids are natural phenolic compounds which appear as secondary metabolites of plants (Ilić, *et.al.*, 2004). The name "Flavonoid" is derived from Greek word "Flavus" which means yellow (Zechmeister, 1957)

A number of flavonoids have anti-protozoal activities (Wright and Phillipson, 1998). Some flavonoids like myricetin and kaempferol-3-glucoside have an anti-HIV-I potency at non-toxic concentration. (Hostettman, *et.al.*, 1995). Some of the minor flavonoids have very interesting activities. They have anti-microbial, anti-fungal and cytotoxic properties. (Harborne, 1988). Tricin has smooth muscle movement activity (Ferguson *et.al.*, 1950). The anthocyanin isorhamnetin of the algae *Chlamydomonas* is a highly potent sex determining hormone (Kuhn, *et.al.*, 1994).

Isoflavonoids act as venom antidote, whereas some flavonoids are used as inhibitors of CCl₄ induced cytotoxicity in isolated hepatocytes. The biosynthesis of coumesterol in leaves is induced by UV light, so its level is used for the indication of damage by UV radiation to the plant system (Harborne and Mabry, 1982; Ruzicka, 1953).

The health effects of flavonoids have long been recognized for antioxidant, anti-inflammatory, anti-allergic, hepatoprotective, antithrombotic and antiviral properties (Middleton and Kandaswami, 2000). Medicinal activities shown for flavonoid compounds range from scavenging of harmful reactive oxygen species, enzyme inhibition, anti-inflammatory, vascular and estrogenic activities to antitumor activities. Anthocyanins and some flavonoids are UV protectants and signaling transmitters. Isoflavones, the bioactive ingredient in leguminous vegetables, not only cause a small reduction in blood cholesterol but also reduce blood pressure and oxidative stress (Anderson and Anthony, 1999).

Acacia nilotica var *nilotica* is found in Burkina Faso, Cameroon, Chad, Egypt, Ethiopia, Mali, Nigeria, Oman, Sudan, Tanzania, Yemen, Zanzibar (Barnes *et al.*, 2002). It is used as effective medicine in treatment of malaria, sore throat (aerial part) and toothache (bark). The powdered bark of the plant with little salt is used for treating acute diarrhea (Mann, *et al.*, 2003). The root is said to cure impotency (James and Duke, 1983). Astringent bark is used for diarrhea, dysentery, and leprosy. Bruised leaves are poultice onto ulcers. The gum or bark is used for cancer of ear, eye, or testicles, indurations of liver spleen. The seeds are also used for cancer, colds, congestion, coughs, diarrhea, dysentery, fever, hemorrhoids, ophthalmia, sclerosis, smallpox, and tuberculosis. Bark, leaves, and young pods are strongly astringent due to tannins and are chewed in Senegal as anti-scorbutic. Bark decoction is drunk for intestinal pains and diarrhea. Other preparations are used for coughs, toothache, ophthalmia, and syphilitic ulcers. In Lebanon, the resin is mixed with orange-flower and used as infusion for typhoid convalescence. Egyptian Nubians believe that diabetics may eat unlimited carbohydrates as long as they also consume powdered pods (Duke, 1983). Extracts of plant were found to be inhibitory to at least four species of human pathogens (Umalkar, 1977).

Materials and Methods:-

Plant material:-

The heartwood of *Acacia nilotica* var *nilotica* was collected from Elobied (western Sudan). The plant was authenticated by the Department of Botany, University of Khartoum.

Methods:-

Extraction of flavonoids:-

Powdered air-dried heartwood (1kg) of *Acacia nilotica* var *nilotica* was extracted with 80% methanol (5L) at ambient temperature for 72hr. The solvent was removed *in vacuo* to give a crude product.

Isolation of flavonoid compounds from heartwood of *Acacia nilotica* var *nilotica*:-

The methanolic extract was mixed with small amount of polyamide and applied on a polyamide (1000g) column. The column was eluted with mixtures of water: methanol (4:1, 3:2, 1:3; v:v) and finally 100% methanol. Fractions of 250 ml were collected and investigated by PC (Whatman No. 3) using three solvent systems: BAW; 15% AcOH and distilled water. Chromatograms were visualized under UV light before and after exposure to ammonia vapor. According to TLC pattern, similar fractions were combined and concentrated to dryness under reduced pressure to obtain main fractions. Detected compounds were isolated by PC from column fraction using acetic acid (15%). The isolated compounds were further purified by re-chromatography on Sephadex LH-20 column using H₂O:MeOH(4:1;v:v), H₂O:MeOH(3:2;v:v) and methanol.

Preparation of bacterial suspensions:-

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto agar slopes and incubated at 37° C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100ml of normal saline to produce a suspension containing about 10⁸ – 10⁴ colony forming units per ml. The suspension was stored in the refrigerator at 4°C until used. The average number of viable organisms per ml of the saline suspension was determined by means of the surface viable counting technique. Serial dilutions of the stock suspension were made in sterile saline in tubes and one drop volumes (0-20ml) of the appropriate dilution were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature to dry, and then incubated at 37° C for 24 hours.

Testing for antibacterial activity:-

The cub-plate agar diffusion method was adopted, with some minor modifications, to assess the antibacterial activity of the isolated compounds. (2ml) of the standardized bacterial stock suspension were mixed with 200ml of sterile molten nutrient agar which was maintained at 45° C in water bath. (20ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes; the agar was left to settle in each of these plates which were divided into two halves. Two cubs in each half (10mm in diameter) were cut using sterile cork borer (No.4). Each of the halves was designed for one of the isolated compounds. Separate Petri dishes were designed for standard antibacterial chemotherapeutic agents (ampicillin and gentamycin).

The agar discs were removed and cups were filled with (0.1ml) sample of each compound using adjustable volume micro liter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37° C for 18 hours.

The above procedure was repeated for different concentrations of the isolated compounds and the standard antibacterial chemotherapeutics. After incubation, the diameters of resultant growth inhibition zones were measured.

Results and Discussion:-

Spectral data of compound (I):-

IR spectrum (Fig.1) showed ν_{\max} (cm^{-1} , KBr disc): 3456 (OH), 1613 (C=O), 1505, 1440 and 1344 (C=C, Ar.), 1108 (C-O), 864, 843, 786 (C-H, Ar., bending).

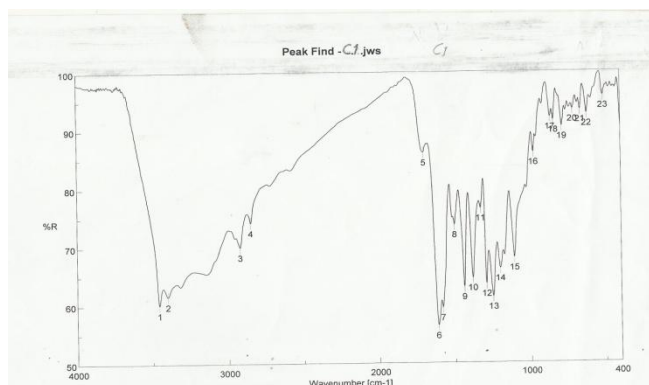


Fig. 1: The IR spectrum of compound I

The UV spectrum (Fig. 2) gave λ_{\max} (MeOH): 249, 347nm which is characteristic of flavones (Mabry *et.al.*, 1970). Addition of NaOMe to a methanolic solution (Fig.3) gave a 49 nm bathochromic shift in band I without decrease in intensity and this is diagnostic of a free 4'-OH (Mabry *et.al.*, 1970). The sodium acetate spectrum (Fig.4) gave a 6nm bathochromic shift at band II which is indicative of a 7-OH function. The boric acid spectrum (Fig.5) revealed a 22nm bathochromic shift in band I. This indicates the presence of a B-ring catechol moiety (Mabry *et.al.*, 1970). When AlCl_3 was added to a methanolic solution of compound I (Fig.6), band I shifted bathochromically by 30nm. The aluminium chloride spectrum degenerated in HCl (Fig.7) indicating a catechol system (Mabry *et.al.*, 1970). This supports the previous findings with boric acid.

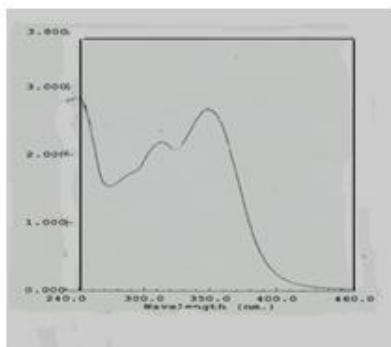


Fig2: UV spectrum of compound I

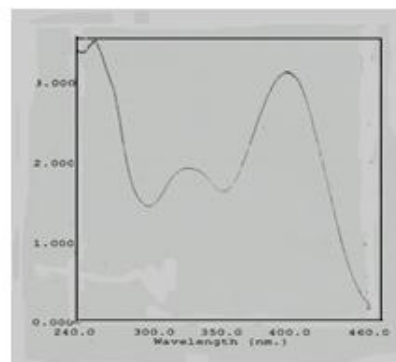


Fig. 3: Sodium methoxide spectrum of compound I

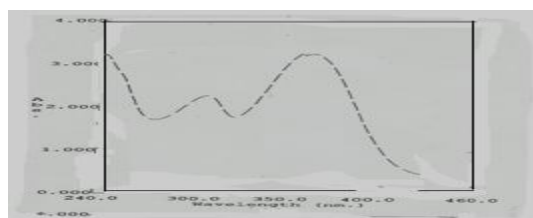


Fig.4: Sodium acetate spectrum of compound I

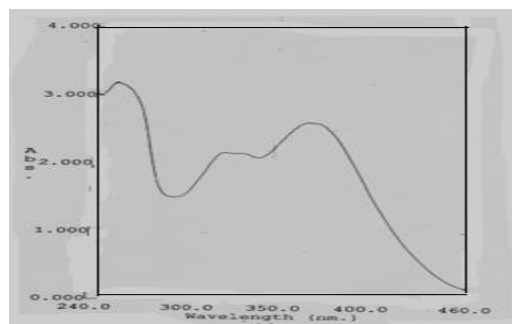


Fig.5: Boric acid spectrum of compound I

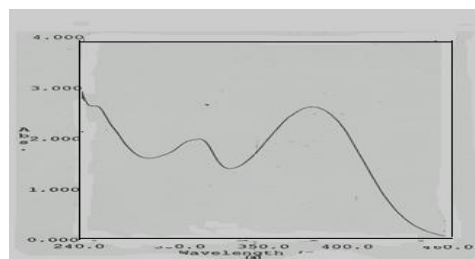


Fig.6: Aluminium chloride spectrum of compound I

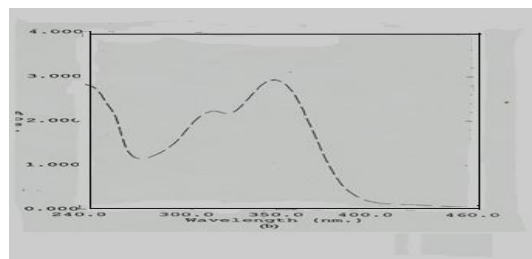


Fig.7: Aluminium chloride/HCl spectrum of compound I

^1H NMR spectrum (Fig.8) showed a doublet at δ 7.8ppm (1H) characteristic of C_5 -proton. The doublets at δ 6.60 and δ 6.8ppm (2H) are characteristic of C_6 - and C_8 - protons respectively, while the doublet at δ 7.5ppm (2H) accounts for the *meta*-coupled C_2 - and C_6 - protons of ring B. The resonance at δ 7.4ppm (1H) is characteristic of C_5 -proton (Mabry *et.al.*, 1970). The singlet at δ 3.7ppm (3H) accounts for a methoxyl function. Due to absence of the signal of C_3 olefinic proton, the methoxyl function was assigned to position 3. The mass spectrum (Fig. 9) gave m/z 300 for the molecular ion.

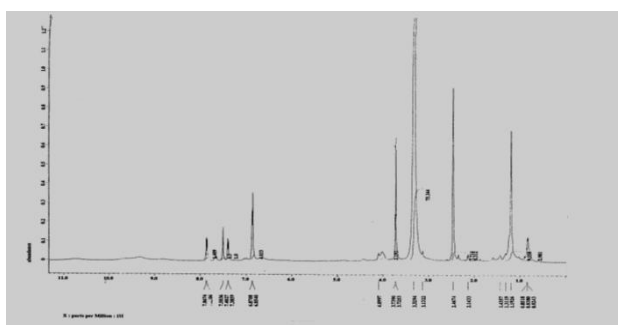


Fig. 8: ^1H NMR spectrum of compound I

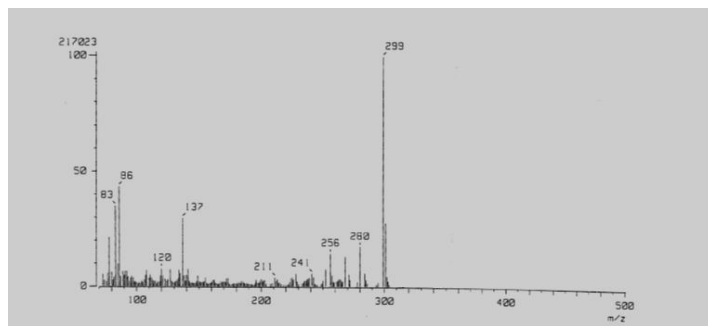
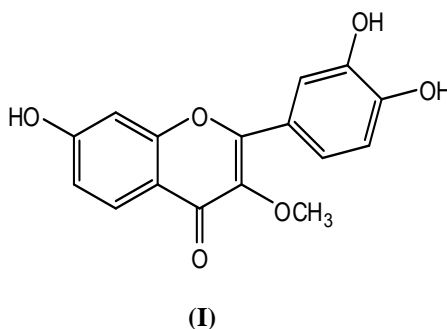


Fig. 9: Mass spectrum of compound

On the bases of the above cumulative data compound I is a: 7, 3', 4'-trihydroxy-3-methoxyflavone.



Spectral data of compound II:-

The IR spectrum of compound II (KBr disc) (Fig.10). showed: ν 3449 (OH), 1611 (C=O), 1568, 1509, 1464 and 1415(C=C, Ar.), 1129 (C-O), 838, 797, 768 cm^{-1} (C-H, Ar, bending)

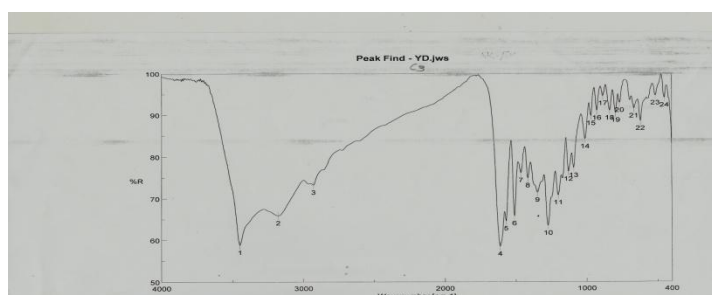


Fig .10: IR spectrum of compound II

Compound II gave UV absorption characteristic flavonols (Fig.11) (Mabry *et.al.*, 1970). It revealed λ_{max} (MeOH) 248, 362 nm. Addition of NaOMe to a methanolic solution of compound II (Fig.12) gave a 45 nm bathochromic shift. Since the spectrum degenerated within 3 minutes, then a 3, 4'-hydroxylation pattern is anticipated. The sodium acetate spectrum (Fig.13) gave a 15 nm bathochromic shift in band II which is indicative of 7-OH function. The boric acid spectrum (Fig.14) revealed a 19 nm bathochromic shift in band 1, indicating the presence of a (B) ring catechol system. When AlCl_3 was added to a methanolic solution of compound II (Fig.15), band I shifted bathochromically by 61nm. This indicates a 3- or 5-OH or an ortho-dihydroxyl system. The spectrum was acid – stable (Fig.16) and the magnitude of the bathochromic shift is indicative of a 3-OH function(Mabry.,*et.al.*, 1970).

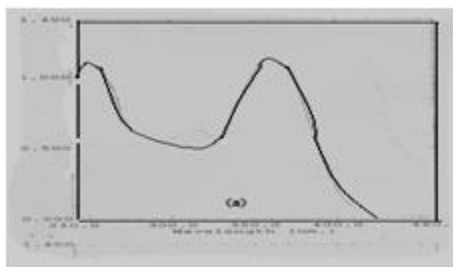


Fig.11: UV spectrum of compound II

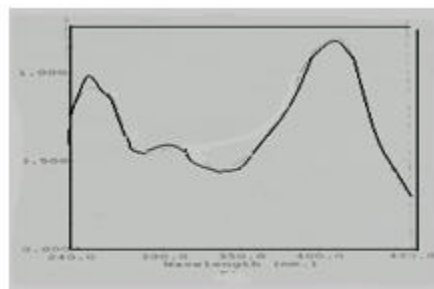


Fig.12: Sodium methoxide spectrum of compound II

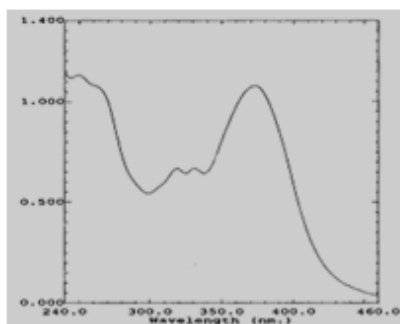


Fig.13: Sodium acetate spectrum of compound II

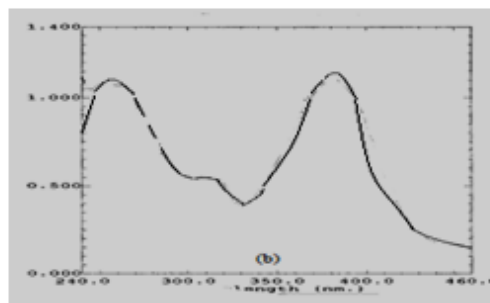


Fig.14: Boric acid spectrum of compound I

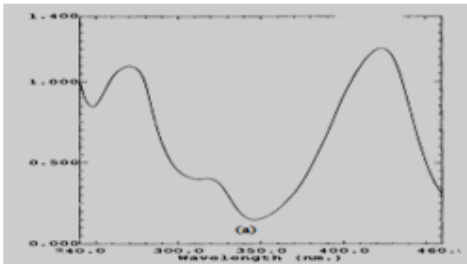


Fig.15: Aluminium chloride spectrum of compound II

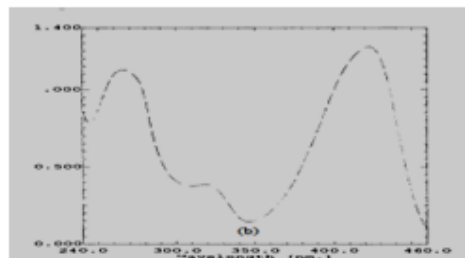


Fig.16: Aluminium chloride/HCl spectrum of compound II

^1H NMR spectrum (Fig.17) showed a doublet at δ 7.8ppm (1H) characteristic of C_5 - proton. This proton resonates well downfield from the rest of (A) ring protons due to the deshielding effect of the 4-keto function. The doublet at δ . 6.8ppm (2H) is characteristic of C_6 - and C_8 protons. The doublet at δ . 7.6ppm (2H) accounts for the *meta*-coupled C_2 - and C_6 -protons of the (B) ring, while the doublet at δ .7.4ppm (1H) was assigned for C_5 - proton. The mass spectrum (Fig. 18) gave m/z 286 for the molecular ion.

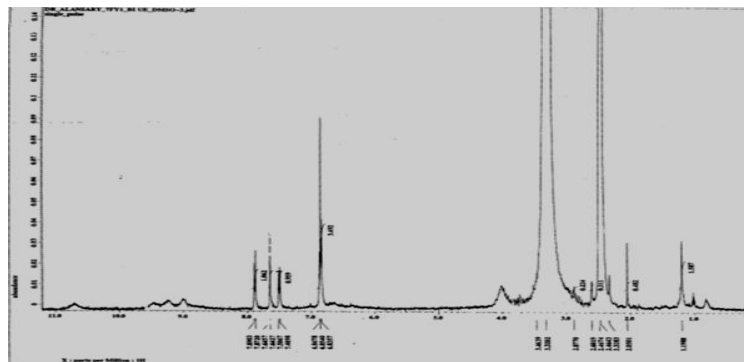
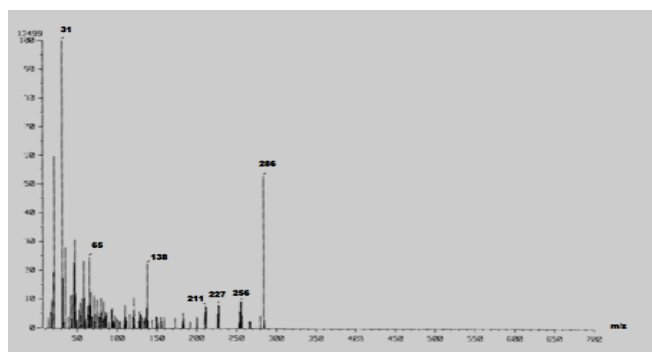
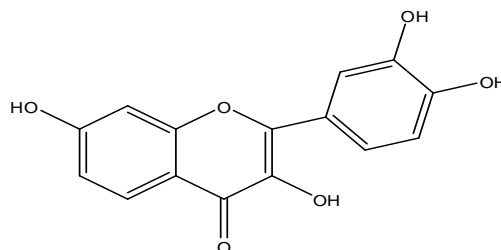
Fig. 17: ¹H NMR spectrum of compound II

Fig. 18: Mass of compound II

On comparing the above cumulative data with literature data, the following structure was suggested for compound II:



7,3',4'-trihydroxyflavonol

The antibacterial activity of the tested compounds (I and II) was examined against Gram positive bacteria (*Bacillus subtilis*, *Bacillus cereus*, and *staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* and *pseudomonas aeruginosa*). The obtained results were compared with reference antibiotics. Table 1 shows that compounds (I) and (II) had different antibacterial responses. Table (2) shows the antibacterial activity of standard antibacterial chemotherapeutic agents against four standard bacteria. The results were interpreted in terms of the commonly used terms (<9mm : inative; 9-12mm:partially active;13-18mm : active;>18mm:very active)

Table 1 :Antibacterial activity of compounds I and II (Conc. 100mg/ml ; M.D.I.Z ,mm)

Bacteria	Gram stain reaction	Control (Methanol)	Compound I	Compound II
<i>Bacillus cereus</i>	Positive	0.0	13	11
<i>Bacillus subtilis</i>	Positive	0.0	21	15
<i>Escherichia coli</i>	Negative	0.0	10	22
<i>Pseudomonas aeruginosa</i>	Negative	0.0	13	22
<i>Staphylococcus aureus</i>	Positive	0.0	20	18

Table 2 : Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Pa.
Ampicillin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

- Sa.: *Staphylococcus aureus*
- Ec.: *Escherichia coli*
- Pa.: *Pseudomonas aeruginosa*
- Bs.: *Bacillus subtilis*

Conclusion:-

Purification of *Acacia nilotica* var *nilotica* extract gave two compounds : (I) and(II). Compound I is a 7, 3', 4'-trihydroxy-3-methoxyflavone, and compound II is a 7, 3', 4'-trihydroxyflavonol.

In the cup plate agar diffusion assay , compounds (I) and (II) were tested against Gram positive bacteria :*Bacillus subtilis*, *Bacillus cereus*, *staphylococcus aureus* and Gram negative bacteria: *Escherichia coli*, *pseudomonas aeruginos* and different antibacterial responses were observed.

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