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Flavone Biotransformation by *Aspergillus niger* and the Characterization of Two Newly Formed Metabolites

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***Aspergillus niger* isolated from *Allium sativum* was used at large scale fermentation (150 mg flavone/200 *ml* medium) to obtain suitable amounts of the products, efficient for identification. Then spectral analysis (UV, IR, ^{1}H-NMR, ^{13}C-NMR) and mass spectrometry were performed for the two products, which contributed to the identification process. The metabolite (1) was identified as 2'-hydroxydihydrochalcone, and the metabolite (2) was identified as 2'-hydroxyphenylmethylketone, which were more active than flavone itself. Antioxidant activities of the two isolated metabolites were tested compared with ascorbic acid. Antioxidant activity of metabolite (1) was recorded 64.58% which represented 79% of the antioxidant activity of ascorbic acid, and metabolite (2) was recorded 54.16% (67% of ascorbic acid activity). However, the antioxidant activity of flavone was recorded 37.50% which represented 46% of ascorbic acid activity. The transformed products of flavone have anti-microbial activity against *Pseudomonas aeruginosa*, *Aspergillus flavus* and *Candida albicans*, with MIC was recorded 250 μg/*ml* for metabolite (2) against all three organism and 500, 300, and 300 μg/*ml* for metabolite (1) against tested microorganisms (*P. aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and *Klebsiella pneumonia*, *Fusarium moniliforme*, *A. flavus*, *Saccharomyces cerviceae*, *Kluveromyces lactis* and *C. albicans*) at this order.**

KEYWORDS : Anit-microbial, Antioxidant, *Aspergillus*, Biotransformation, Flavone

Biotransformations were observed by humans well before they were appreciated. Microbial transformations have long been valued for the production of nearly every class of steroid hormone product (Smith, 1973). The successful application of microbial transformation as an early form of biotechnology is widely accepted in the industrial preparation of hormones. The important reactions catalyzed by microbial enzymes include dehydrogenation, side-chain cleavage, and hydroxylation at the strategic positions 11α-, 11β-, 17α- and 21 of the steroid nucleus.

Degradation of flavonoids, rutin, and phloridzin in particular by various bacteria and molds has been reported (Cheng *et al*., 1969; Barz, 1970). Flavonoids are the major red, blue, and purple pigments in plants, which have a considerable medical importance. The degradation usually involves an initial release of the sugars by intracellular glycosidases, followed by hydrolytic cleavage of the heterocyclic ring of the aglycone (Krishnamurthy *et al*., 1970).

Screening of 5-hydroxyflavone with several microorganisms showed the formation of several metabolites, which were produced in relatively small amounts (Ibrahim and Abul-Hajj, 1989). However, incubations with *Streptomyces fulvissimus* showed complete transformation of 5'-hydroxyflavone within 48 hr. The initial metabolites isolated after 48 h were identified as 5,4'-dihydroxyflavone and 5,3',4'-trihydroxyflavone. Longer incubations resulted in a decrease in the amount of 5,4'-dihydroxyflavone which was associated with an increase in the formation of a metabolite that was very polar.

Rhizobia can catabolize polycyclic flavonoids responsible for nod gene induction (Cooper *et al*., 1995). Studies of flavonoid metabolism by free-living rhizobia (*Rhizobium meliloti* and *Bradyrhizobium japonicum*) were reported. A wide range of flavonoid and isoflavonoid nod gene inducers could be catabolized by rhizobia via c-ring fission mechanisms. The products included both open and closed c-ring modification structures as well as monocyclic hydroaromatics. The process could be seen as a reversal of plant flavonoid biosynthesis. The metabolic fate of naringenin during nod factor synthesis by *R. leguminosarum bv. Viciae* was studied. Naringen was shown to be assimilated by *Rhizobium* when it was presented at nod gene inducing concentrations.

Isolation and synthesis of isoflavonoids have been frequently attempted, due to their interesting biological activities. Greene (1995) mentioned that flavonoids have antioxidant activity which play an important part in detoxification of free radicals, which accumulate in patients body, causing cancer, and many cardiovascular disorders. The introduction of hydroxyl groups into isoflavonoids by the use of enzymes is an alternative to conventional chemical synthesis, (Seeger *et al*., 2003). The hydroxylations by biotransformation of ring B of isoflavonoids are

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likely to improve their antioxidant properties (Briviba *et al.,* 1997 and Arora *et al.*, 1998).

Kim *et al.* (1998) tested the biotransformation of some flavonoids (Rutin, hesperdin, and naringin) using intestinal microflora to produce phenolic acids. The cleavage of flavonoids by intestinal microflora makes understanding of the mammalian metabolism of these natural products complicated. Indeed, more than 40 mammalian metabolites have been identified, many of which are hydroxylated at 3 and 6 positions (Das *et al.*, 1973).

Microbial transformation of flavones of *Psidia arabica* by *Cunninghamella elegans* producing the 3'-glucoside conjugates of the flavones was reported (Ibrahim *et al.*, 1997). Sulfations of naringenin by *Cunninghamella elegans* to form naringenin-7-sulfate were reported (Ibrahim, 1999).

Hunter (1995) has reported anti-inflammatory and anti-allergic effects of flavonoides on human. Because flavonoid are consumed in appreciable amounts in our diet, knowledge of their pharmacological and physiological properties is of significant importance. Therefore, we have planned to carry out the present study to test the possibility of getting new transformation products that might have a medical importance to human.

Materials and Methods

Initial screening for flavone biotransformation using some of the isolated fungi. Initial screening has been carried out using 24 different fungal species that were isolated from 44 medicinal plants at Kafr El-Sheik governorate, Egypt (data not shown). The fungal genera which were selected in initial screening for flavone transformation were; *Aspergillus*, *Fusarium*, *Alternaria*, *Stemphylium*, *Macrosporium*, *Nectria*, *Hormodendrum*, *Trichothecium*, *Spicaria*, *Microdochium*, *Gibberella*, *Macrophomina*, *Athelia*, and *Cladosporium*. Detection of flavone metabolites was performed using silica gel plate coated with (60GF254) through thin layer chromatography (TLC).

Fermentation technique. Fermentation liquid cultures were initiated by transferring about 5 *ml* of fungal suspension (two-week old slants) into 50 *ml* sterile liquid medium contained in 250 *ml* Erlenmeyer flasks placed on a shaker operating at about 150 rpm at 27°C for 48 hr (stage 1).

At stage 2, cultures were obtained by transferring about 5 *ml* of stage-1 culture to 250 *ml* Erlenmeyer flasks containing 50 *ml* of the same liquid medium at the same conditions. Cultures were allowed to grow for 24 hr before adding of substrate (flavone) dissolved in N,N-dimethyl formamide (DMF) at a concentration of 5 mg/50 *ml* medium, and continued to incubate at the same conditions for 6 days. One flask was used as control in which the culture was allowed to grow under the same conditions without addition of fungal spores (Ibrahim and Abul-Hajj, 1990).

Fermentation liquid medium was composed of 10 g glucose, 5 g peptone, 5 g yeast extract, 5 g sodium chloride, 5 g K_2HPO_4, 10 *ml* glycerol per 1 liter, and pH was adjusted to 6 before autoclaving.

Detection of transformation products. Sampling of the culture was carried out by taking 5 *ml* of culture suspension and then extracted with 5 *ml* chloroform. Then, Chloroform extract was evaporated and the residue was dissolved in a few drops of methanol, then 20 μl was spotted into silica gel plate (60GF254) (Ciegler *et al.*, 1971).

Isolation and Detection of transformation products. A commercial applicator for spreading the prepared slurries of silica gel (adsorbent) as thin layer to glass plates is usually 5 × 20 cm or 20 × 20 cm. Adsorbent was applied as aqueous slurries (5 g silica gel/20 *ml* dist. Water). The thickness of the film was 250 μm. Samples which prepared from the fermentation culture in the previous step were applied. Then the applied spots were eluted with methanol-water-acetic acid (55 : 45 : 0.5, V/V/V) and/or chloroform-methanol (20 : 1, V/V) were being used in this work. Chromatographic plates were usually developed once by the ascending technique, at room temperature, to a height of 15~18 cm. The separated bands on thin layer of silica gel were detected using two detection methods. The plates were dried and visualized under UV-light or sprayed with sulphuric acid or exposed to ammonia vapors (Horowitz, 1957).

Large scale fermentation technique and products purification. *A. niger* isolated from *A. sativum* (data not shown) was used as transfomant in this technique. The same procedures mentioned above were used to obtain large amounts of products sufficient for spectroscopic analysis. Greater concentrations of flavone (150 mg flavone/200 *ml* medium) were prepared to add when large containers (flasks) were used in fermentation technique as a way for obtaining large amount of products. *A. niger* was used for large scale fermentation in order to transform flavone. About 150 mg flavone/200 *ml* medium in one liter flasks was used in stage 2 cultures. The fermentation was terminated after 6-days starting with addition of flavone. The cells were separated from the medium by filtering through a cheese cloth. The cells were extracted with ethyl acetate containing 1% methanol, while the filtrate was extracted by chloroform containing 1% methanol. The combined extracts were evaporated under vacuum.

Isolation of flavone metabolites. The concentrated fermentation extracts were adsorbed on a suitable amount of

silica gel using thin layer chromatographic technique. Elution was carried out using chloroform : water : acetic acid (55 : 45 : 0.5 V/V/V) and chloroform-methanol mixtures (20 : 1 V/V). Then spots with similar flow rate were scrapped and combined to dissolve in methanol which centrifuged to separate the filtrate. Methanol was evaporated to obtain the products. Identification of the obtained products were achieved using spectroscopic analysis (UV, IR, ^{1}H-NMR, ^{13}C-NMR) at Faculty of Science, Alexandria University, and Mass spectrum at Faculty of Science, Cairo University.

Spectroscopic analysis for identification of the isolated metabolites UV analysis. UV analysis was carried out with Perken Elmer Lambda 4B UV/vis spectrophotometer.

IR analysis. Activities of analyzed samples in IR were achieved by 1340 Ratio Recording Infrared spectrophotometer.

NMR (Nuclear Magnetic Resonance) analysis. ^{1}H-NMR and ^{13}C-NMR measurements were carried out with a Jeol JNM ECA 500-MHZ. A sample changer was used to perform one-dimensional NMR scans for 1-h time periods. All of the samples were dissolved in deuterated DMSO (DMSO-d6). The chemical shifts were expressed as δ values (parts per million) by using the solvent as an internal reference (DMSO-d6: $\delta H = 16.22$, $\delta c = 77.40$). The spectral width was set at 200 ppm. Deuterated water was used to blank water peaks.

Mass spectral analysis. PE Sciex API300 LC/MS, single-quadrupole instrument (Toronto, Ontario, Canada) equipped with an electron spray ion source was used for analysis of masses of the breakdown products. All MS analyses were carried out in the positive ionization mode. The ring voltage was 150 V, and the mobile phase was a gradient of 1 : 1 acetonitrite-water containing 1 mM ammonium acetate. Spectra were analyzed and interpreted using BioMultiview program (PE Sciex).

Biological evaluation of flavone transformation products. Flavone and its metabolites were tested for their antibacterial and antifungal activities. Four different bacterial species were obtained from culture collection of Faculty of Pharmacy, Tanta University, namely; *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* 418, and *Klebsiella pneumonia*. Two different fungal species; *Aspergillus flavus* and *Fusarium moniliforme* and three yeasts were; *Saccharomyces cervisieae*, *Klyvromyces lactis*, and *Candida albicans*.

Standard antibacterial (antibiotic) discs containing Streptomycin were obtained from El-Gomhorya Co. Tanta, Egypt, and clotrimazole (10 mg/*ml*) was obtained from El-Nasr Co. Cairo, Egypt as antifungal agent. Stock solutions of transformation product-1 and product-2 (50 μg/*ml*) were prepared for disc impregnation. Sabouraud dextrose broth and agar were prepared. Also Nutrient broth and Nutrient agar were prepared to grow the different microorganisms.

Biotransformation products were obtained from the extracts which were purified as a result of transformation of flavone using *A. niger* (isolated from *Allium sativum*). Both Flavone transformation product (1) and Product (2) as well as flavone as standard antimicrobial activity were evaluated.

Disc preparation. A solution of 50 μg/*ml* of each metabolite was prepared for antifungal, anticandida and antibacterial assays. Filter paper discs (4 mm diameter) were impregnated in each solution, and then air dried at room temperature.

Sreening for antimicrobial activity using disc diffusion method. The stock cultures of the tested organisms were inoculated into Sabouraud-dextrose broth for 4-days at 28°C (for fungi) or inoculated into nutrient broth for 24 hrs at 37°C (for bacteria). Prepared culture suspensions were inoculated and spread on the surface of agar plates containing sabouraud's media for fungi and yeast, while nutrient agar media were used for bacteria Filter paper discs (4 mm diameter) which were impregnated in each solution (flavone and metabolites), Streptomycin discs (antibiotic, 30 μg/disc), and clotrimazole discs (antifungal, 10 mg/*ml*) were distributed at the surface of the agar plates. The plates were incubated for 24 hrs at 28°C (for fungi and yeast), and at 37°C (for bacteria, then taken for investigation. Antimicrobial activity of flavone and transformed flavone against the tested microorganisms were detected by the presence of inhibition zone around the filter paper discs. The degree of antimicrobial activity was detected by diameter of inhibition zone (Zheng *et al.*, 1996).

Quantitative antimicrobial assay using minimum inhibitory concentration (MIC). Serial concentrations of flavone and transformed flavones (10, 5, 2.5, 1.25, 0.62 mg/*ml*) were prepared (Weidenborner and Jha, 1997).

Cultures which have sensitivity against transformed flavone (using disc diffusion method) were incubated in 5 *ml* broth tubes containing different concentrations of the tested substances. Incubation allowed for 4-days at 28°C (fungi), and at 37°C for 24 hrs (bacteria). Growth was measured by optical density at 660 nm. Minimum inhibitory concentration (MIC) of flavone and its metabolites were detected against each microorganism which selected after detection by disc diffusion method. Minimum inhibitory concentration was the lowest concentration of the antimicrobial substance which inhibits the growth of the tested microorganism.

Antioxidant activity of transformation products of flavone. A method for the screening of antioxidant activity is according to decolourisation assay (Re *et al.*, 1999) that is applicable to both lipophilic and hydrophilic antioxidants, including flavonoids. The method was based on the evaluation of the free-radical scavenging capacity of flavonoid, by addition of H_2O_2 and peroxidase (Cano *et al.*, 1998), whose neutralization was easily followed by reading the decrease in absorbance at 414 nm after the addition of the antioxidants (flavonoids). This assay is similar to that described by Rice-Evans and Miller (1994). Standard solutions of 5.7 mM L-ascorbic acid (Aldrich, Germany) in deionized water and 10 mM TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich, Germany) in methanol were prepared. Diluted samples in water were prepared and used on the day of preparation, while the ascorbic acid solutions were used within 1 hr of preparation. Fifty microlitre of diluted standard (or sample) was mixed in an Eppendorf tube with 950 μl of the free-radical solutions. These solutions were left to react for 10 min. under continuous stirring. The changes in absorbance were then measured at 25°C. The influences of both the concentration of flavonoids and duration of reaction on the inhibition of the radical cation absorption are taken into account. The experiments were carried out using an improved ABTS (2',2'-azinobis-3-ethylbenzothiazoline) decolourisation assay. Antioxidant activity of flavonoids was detected as compared with ascorbic acid (as ideal antioxidant). Percentage of antioxidant activity was determined from the equation: inhibition % = Abs (control) – Abs (test)/Abs (control) × 100.

Results

A. niger transformation of flavone has been traced in order to get an information about the nature of the formed products. Three products were obtained; two of which have been followed by spectroscopic analysis and the other one has been neglected due to its minute amount.

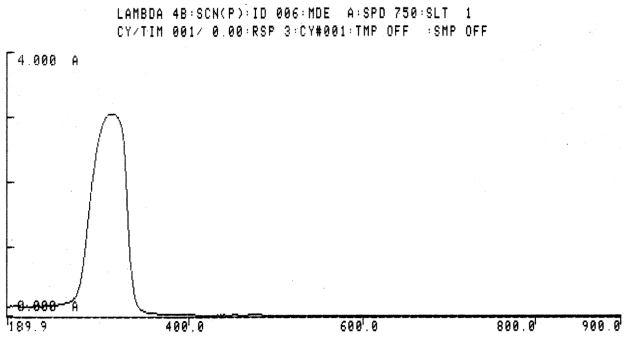


Fig. 1. UV spectrum of metabolite (1).

Chemical characterization and identification of metabolite (1). Metabolite (1) was produced from transformation of flavone by *A. niger*. Its UV spectrum λ-max is 305.4, 278.7, 250.2 nm (Fig. 1). Infrared (KBr) showed absorption at 3665, 2852, 1692, 1553, 1535, 1518, 1483, 1448, 1333, 875, 679 cm^{-1} (Fig. 2). ^{1}H-NMR (DMSO): 4.10 (C-2), 3.68 (2H, C-3), 8.07 (C-5), 7.57 (C-6), 7.67 (C-7), 7.67 (C-8), 8.37 (OH proton) (Fig. 3). ^{13}C-NMR (DMSO): 195.0 (C-4), 163.33 (C-9), 156.28 (C-4'), 132.40 (C-6'), 132.40 (C-2'), 132.10 (C-1'), 129.76(C-7), 129.73 (C-5), 129.70 (C-6), 126.92 (C-10), 119.12(C-3'), 119.12(C-5'), 67.97(O-CH_3), 63.50 (C-3), 39.71 (C-2) (Fig. 4). Mass spectrum (direct probe): m/e 272 (M+), 141, 116, 113, 102, 93, 66, 51 (Fig. 5).

Chemical characterization and identification of metabolite (2): Metabolite (2) was produced from transformation of flavone by *A. niger* isolated from *A. sativum* as well as by other isolated fungi (data not shown). Its UV spectrum λ max is 433, 327, 295 nm (Fig. 6). Infrared (KBr) showed absorption at: 1709, 3639, 2851, 1552, 1536, 1517, 1413, 1333, 873, cm^{-1} (Fig. 7). ^{1}H-NMR (DMSO), 2.95 (^{1}H, t, C-2), 3.57 (^{1}H, C-3), 8.58 (^{1}H, d, C-5), 8.07 (^{1}H, t, C-6), 8.38 (^{1}H, t, C-7), 8.30 (^{1}H, d, C-8, = 9 HZ), 8.88 (OH proton) (Fig. 8). ^{13}C-NMR: 205 (C-4), 163.88 (C-9), 129.80 (C-5), 129.80 (C-7), 118.90 (C-6),

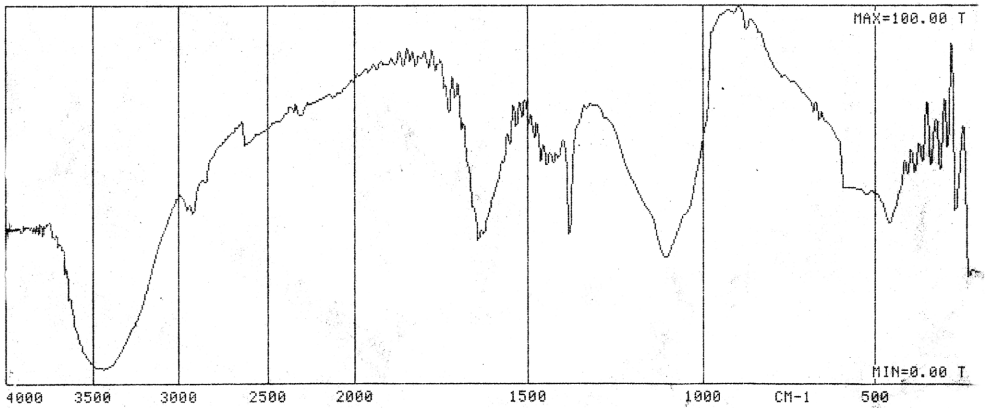


Fig. 2. IR spectrum of metabolite (1).

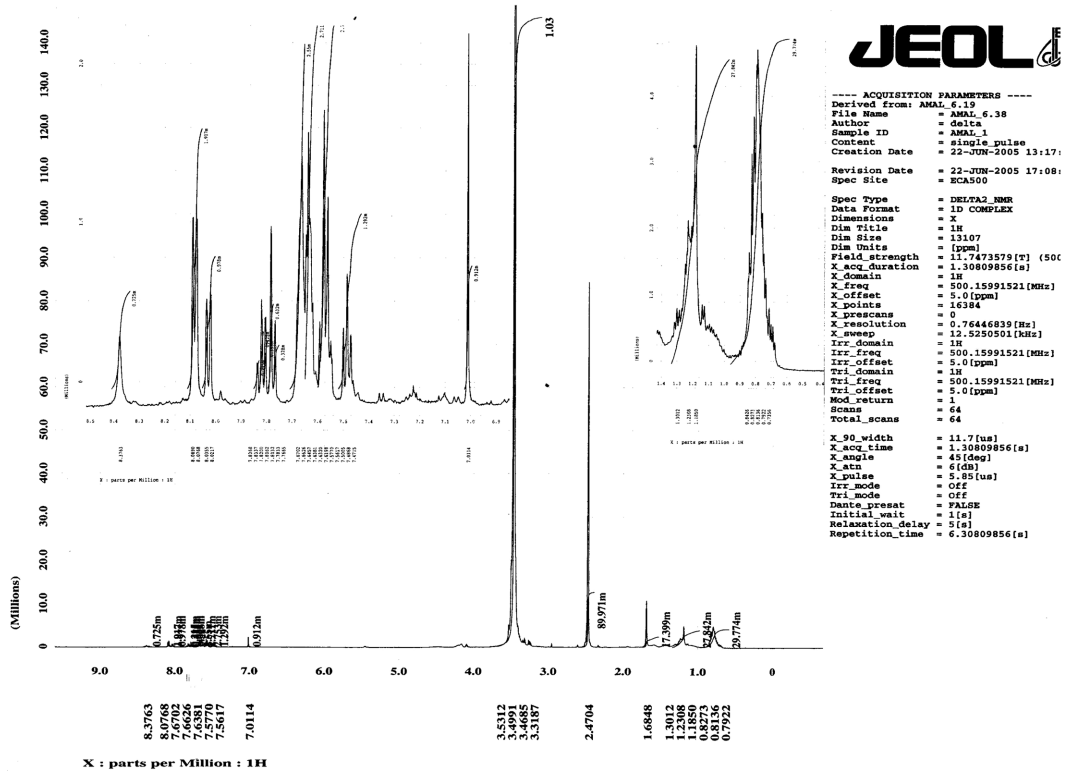


Fig. 3. [1]H-NMR spectrum of metabolite (1).

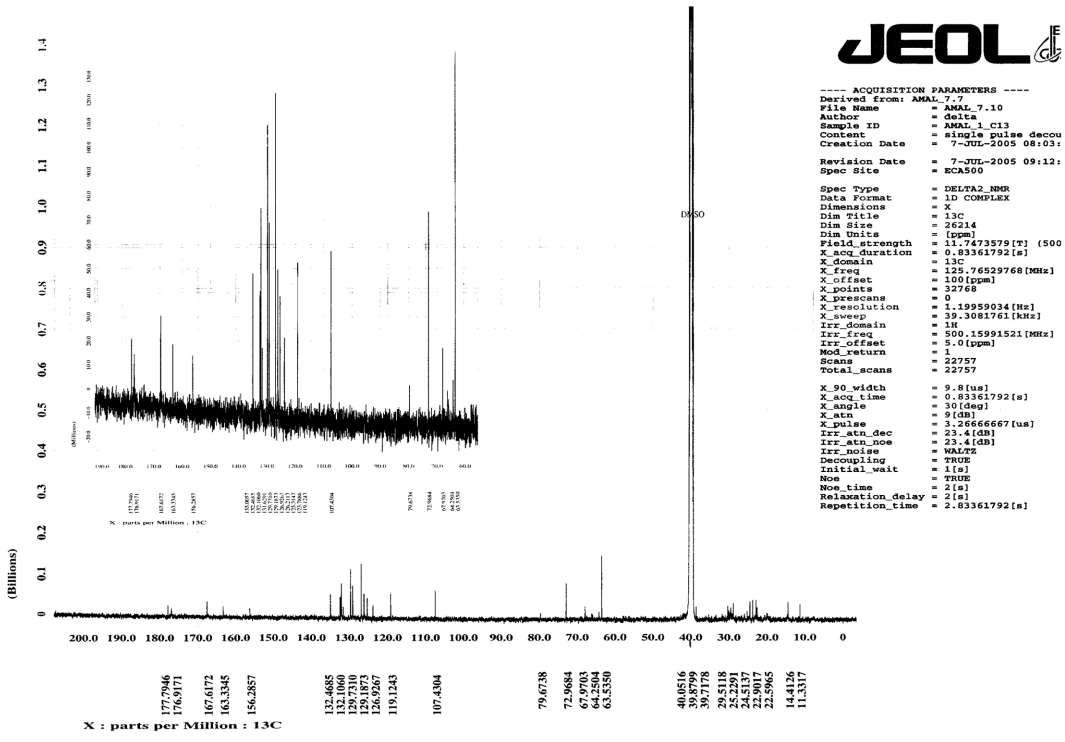


Fig. 4. [13]C-NMR spectrum of metabolite (1).

118.90 (C-10), 118.81 (C-8), 40.00 (C-3), 29.20 (C-2) (Fig. 9). Mass spectrum (direct probe): m/e 150 (M^{+1}), 149 (M^{+1}), 92, 91, 77, 57, 56, 51 (Fig. 10). All the data of either [1]H or [13]C-NMR of Flavone or transformed product one and/ or transformed product two has been summarized in Table 1 and 2).

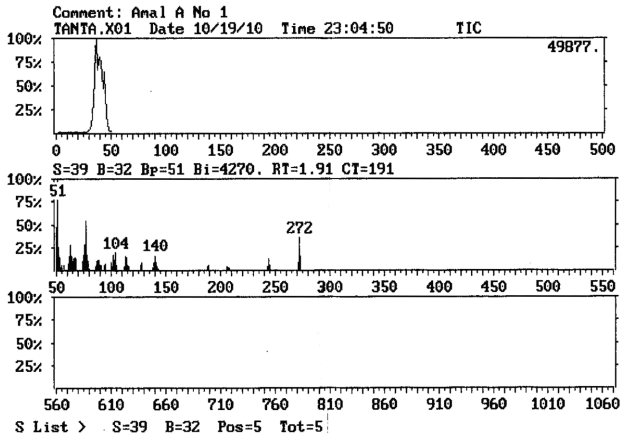


Fig. 5. Mass spectrum of metabolite (1).

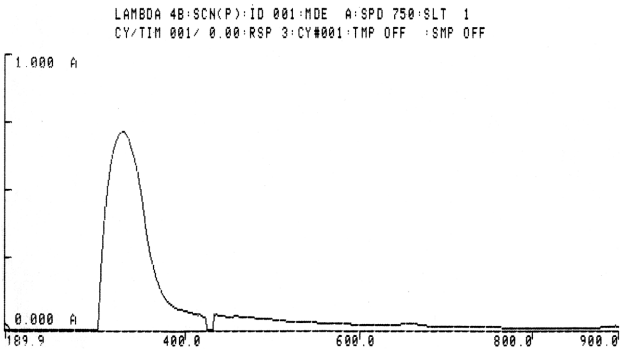


Fig. 6. UV spectrum of metabolite (2).

Biological evaluation of the obtained metabolites. Antimicrobial (antibacterial and antifungal) activity was studied for starting flavone material and two of its transformed and unidentified products (Fig. 11 and Table 3). Four different bacterial species, two fungal species, and three different species of yeast were used for detection the antimicrobial activity. Flavone and its metabolites have antibacterial activity against *P. aeruginosa*, and antifungal activity against *A. flavus*, and *C. albicans*. The transformed and unidentified products have antimicrobial activity more than starting flavone. Product 2 had the greatest antimicrobial activity when compared with product 1 or flavone itself (Zheng *et al.*, 1996).

Antimicrobial activity of the obtained metabolites. Microbes which inhibited by flavone and transformation products were tested for minimum inhibitory concentration (Table 4). Transformation product (2) had the greatest antimicrobial activity which inhibited the growth of *P. aeruginosa*, *A. flavus*, and *C. albicans* at a concentration of 250 $\mu g/ml$.

Antioxidant activity of flavone and transformed flavone. Metabolite(1) and (2) were tested for their antioxidant activities. Antioxidant activity of transformation product (1) was detected as 64.58% (the greatest activity), while detected as 54.16% for product 2, and 37.5% for flavone, and detected as 81.25% for ascorbic acid as referred standard (Table 5).

Discussion

Flavones are a group of multi-ring hydroxyl-containing compounds that are being studied widely for their nutritional value and their use in preventive health care measures. These compounds are found in products as diverse as Ginkgo Biloba, orange juice, and in garden herbs such as dill, oregano and parsely.

Efficient utilization of flavonoids can lead to a positive selection of the utilizers. Therefore it is not surprising that flavonoid-degraders have been reported (Krishnamurthy *et al.*, 1970; Jeffrey *et al.*, 1972; Sakai, 1977; Winter *et al.*, 1991; Rao and Cooper, 1994). Due to their rich carbon contents, flavonoids have potential significance as nutritional sources, whereas their complex structures allow them to have diverse pharmacological and physiological effects (Middle-

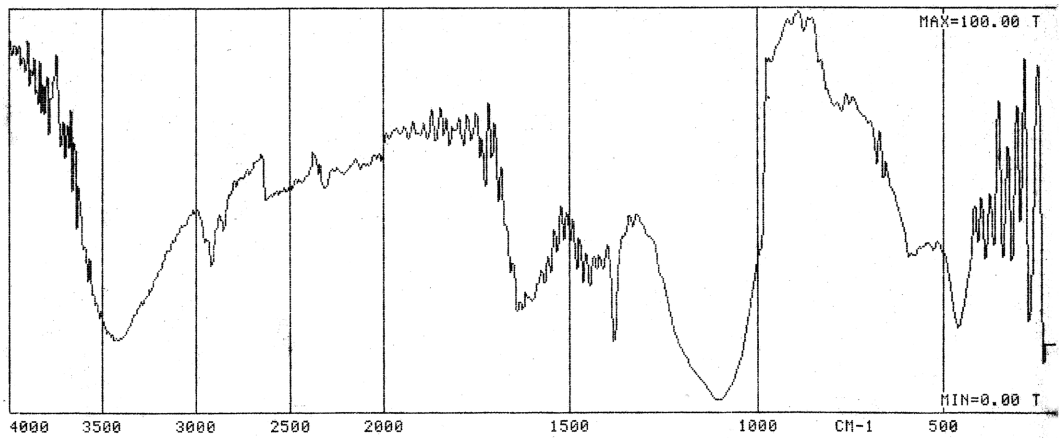


Fig. 7. IR spectrum of metabolite (2).

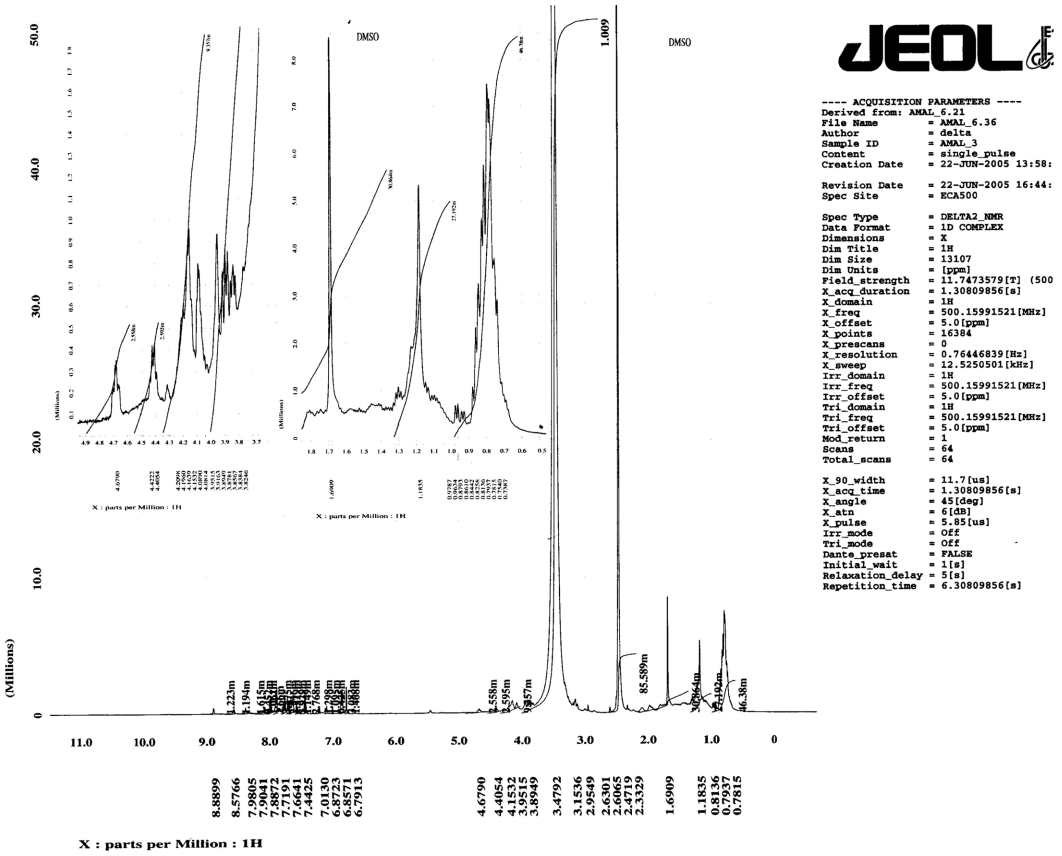


Fig. 8. [1]H-NMR spectrum of metabolite (2).

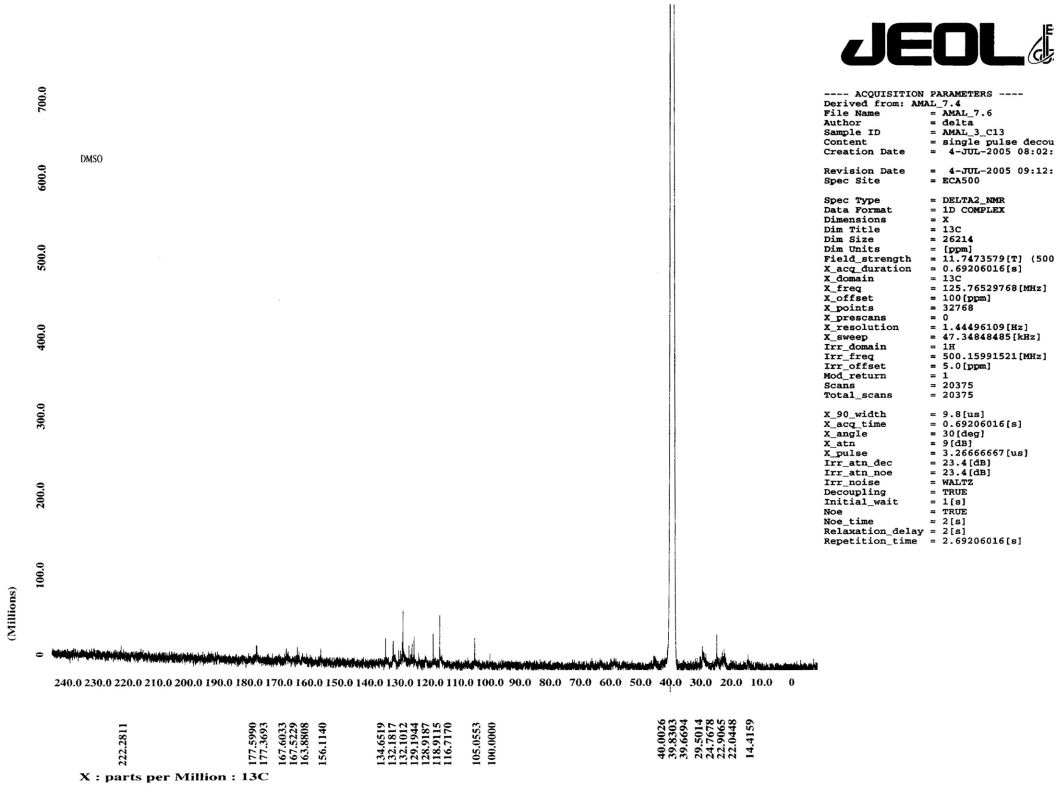


Fig. 9. [13]C-NMR spectrum of metabolite (2).

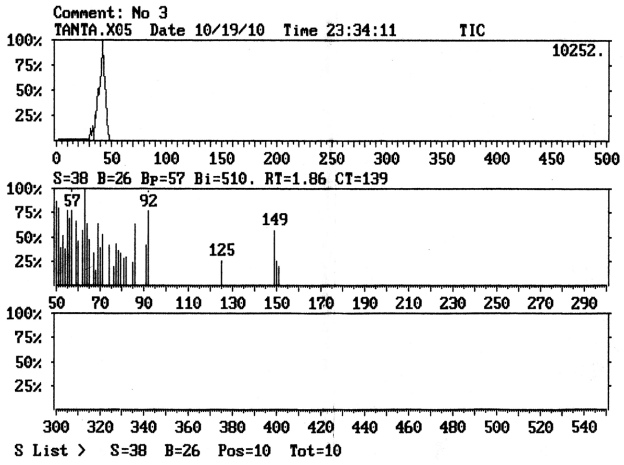


Fig. 10. Mass spectrum of metabolite (2).

Table 2. ^{13}C-NMR analysis of flavone and transformed products

C-atom	Flavone	M1	M2
2	163.80	39.71	29.30
3	105.05	63.50	40.00
4	177.00	195.00	205.00
5	129.19	129.73	129.00
6	129.19	129.70	118.40
7	132.18	129.76	129.80
8	118.10	129.10	118.81
9	163.80	163.33	163.88
10	124.10	126.92	118.90
1'	132.10	132.10	–
2'	128.90	132.40	–
3'	129.10	119.12	–
4'	132.10	156.28	–
5'	129.10	119.12	–
6'	128.10	132.40	–
OCH_3	–	67.97	–

ton and Kandaswami, 1993). The degree of activity for flavone transformation was different with different fungal species, and also different among different fungal isolates under the same species. This was supported when *A. flavus*, *A. niger*, *Fusarium lateratium*, *Alt. tenuis* & *geophila* were used as biotransformants of flavone (data no shown).

Hawksworth and Rossman (1987) estimated as many as 1 million different fungal species, yet only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chapela (1994) estimate there may be at least 1 million species of endophytic fungi alone (Dreyfuss, 1994). Novel microbes usually have associated with them novels natural products. This fact alone helps eliminate the problems of duplication in compound discovery.

Identification of metabolites using spectroscopic analysis. During the course of this study, many fungi were able to hydroxylate ring-B and C to form one or more of most active metabolites (Fig. 11). The identity of these metabolites was established on the basis of different spectroscopic techniques, IR compared to the substrate. The infrared spectra of these metabolites are characterized by broad characteristic for hydroxyl groups. Mass spectra of flavone usually show the characteristic retro-Diels-Alder fragments (Fig. 12).

The retro-Diels-Alder fragments are very helpful in distinguishing the site of the hydroxylation. Ring B hydroxylation is marker by an increase of 16 mass units in case of 4'-hydroxy metabolites or an increase by a mass of 32 in case of 3',4'-dihydroxy metabolites. The molecular ion peaks of these derivatives are very intense and diagnostic (Bowie and Cameron, 1966).

The 4' and 3',4' hydroxylated flavone are not frequently present in natural sources (such as plants) except for 3',4'-dihydroxyflavone (Harborne, 1968). Thus microbial transformations can be extremely helpful in obtaining these compounds. Because many organisms that produce high yields of 4'-hydroxy-flavone were not such

Table 1. ^{1}H-NMR of flavone and its metabolites

C-atom	Flavone	Metabolite (1)	Metabolite (2)
2	7.95	4.10	2.95, t
3	6.95 (2H, d, J = 8.50)	3.68	3.57
4	–	–	–
5	6.95 (2H, d, J = 8.50)	8.07	8.58, d
6	7.95	7.57	8.07, t
7	–	7.67	8.38, t
8	–	7.67	8.30 d, J = 9
9	–	–	–
10	–	–	–
1'	–	–	–
2'	–	7.10, d, J = 8	–
3'	7.76	6.76, d, J = 8.50	–
4'	7.76	-	–
5'	7.45 (1H, t)	6.76, d, J = 8.50	–
6'	8.05 (1H, d, J = 8.16)	7.10	–
OH	–	8.37 (S)	8.88 (S)
OCH_3	–		

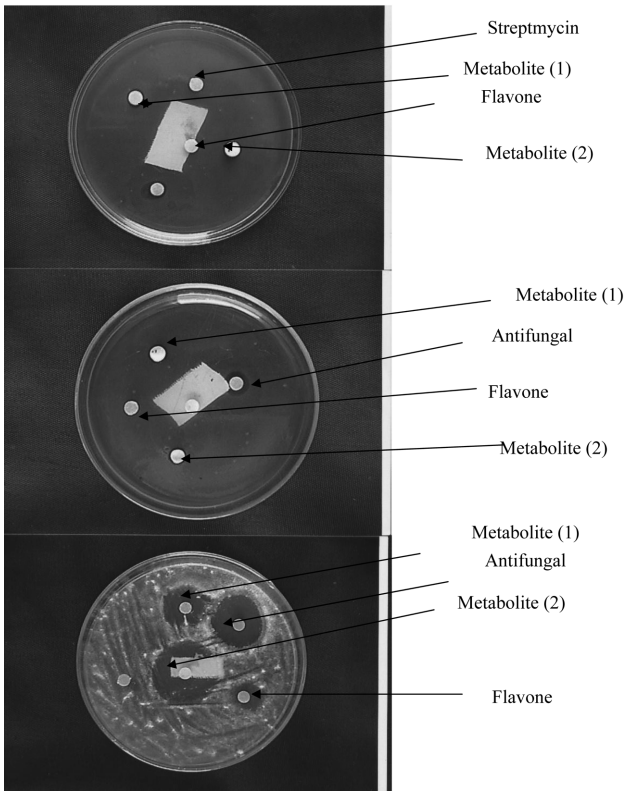


Fig. 11. Antimicrobial activities of flavone and the two produced metabolites. Where A; Antimicrobia activity of *Pseudomonas aeruginosa*. B; Antimicrobia activity of *Candida albicans*. C; Antimicrobia activity of *A. flavus*.

Table 5. Antioxidant assays of transformed flavone by (ABTS) method

Compounds	ABTS Abs(control) − Abs(test)/Abs(control) × 100	
	Absorbance of samples	% inhibition
Control (ABTS)	0.48	0
Ascorbic acid	0.09	81.25
Metabilite (1)	0.17	64.58
Metabolite (2)	0.22	54.16
Flavone	0.30	37.50

good producers of 3',4'-dihydroxyflavone, a different enzyme system seems to be involved in the conversion to 3',4'-dihydroxylated metabolite than that which causes the initial formation of 4'-hydroxylation product.

The site of aromatic hydroxylation by fungi may be predicted, as is the case in mammalian systems, by the rules of electronic substitution reactions. While the electron rich rings are readily hydroxylated (Smith and Rosazza, 1975). These findings may be used to explain the predominance of ring B hydroxylation in flavonoids. Recent studies have showed that mammals such as rats and guinea pigs metabolize flavone to 4'-hydroflavone and 3',4'-dihydroyflavone (Svardal *et al.*, 1981). These results are essentially similar to those obtained by other microbial systems.

Microbial cleavage of flavone by *A. niger*. Several microorganisms were found to be capable of cleaving ring C of flavone. The two ring-C cleavage products were

Table 3. Antimicrobial activity of flavone and some derivatives assayed by disc diffusion method (Inhibition zone diameter, mm) (Weidenborner and Jha, 1997)

Microorganism	Metabolite (1)	Metabolite (2)	Flavone	Standard antifungal (Clotrimazole)	Standard antibacterial (Streptomycin)
Pseudomonas aeruginosa	9.0 ± 2.0	9.6 ± 1.52	6.6 ± 0.57	–	8.0 ± 1.0
Escherichia coli	ND	ND	ND	–	15.30 ± 2.50
Bacillus subtilus	ND	ND	ND	–	15.0 ± 1.15
Klebsiella pneumonia	ND	ND	ND	–	7.0 ± 1.0
Aspergillus flavus	21.60 ± 2.08	26.60 ± 1.50	12.30 ± 1.15	26 ± 1.70	–
Fusarium moniliforme	ND	ND	ND	8.30 ± 1.15	–
Saccharomyces cerviceae	ND	ND	ND	10.60 ± 2.08	–
Kluvromyces lactis	ND	ND	ND	10.60 ± 1.0	–
Candida albicans	14.0 ± 2.0	15.60 ± 2.0	6.60 ± 0.6	22 ± 2.0	–

Where; ND means Not Determined.

Table 4. Minimum inhibition concentration (MIC, $\mu g/ml$) of flavone and its transformed products by tube dilution method

Microorganism	Metabolite (1)	Metabolite (2)	Flavone	Streptomycin sulphate (standard antibacterial)	Clotrimazole (standard antifungal)
Pseudomonas aeruginosa	300	250	500	–	–
Aspergillus flavus	300	250	500	–	62
Candida albicans	400	250	500	–	125

*Reading is the average of three replica.

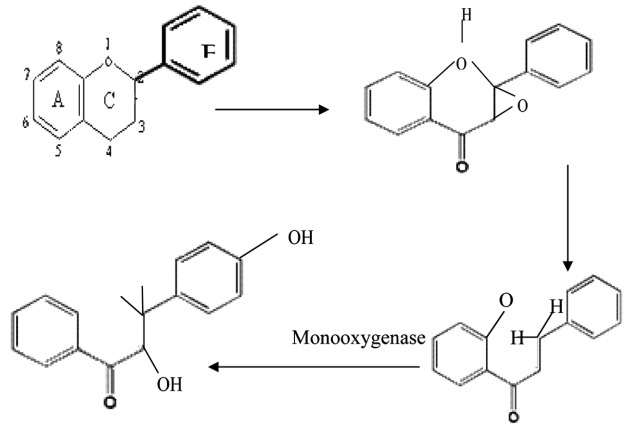


Fig. 12. Microbial hydroxylation of flavone.

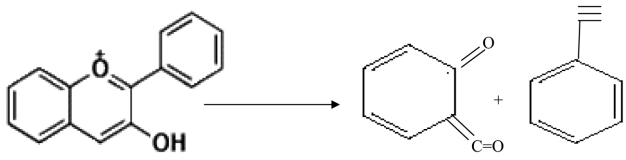
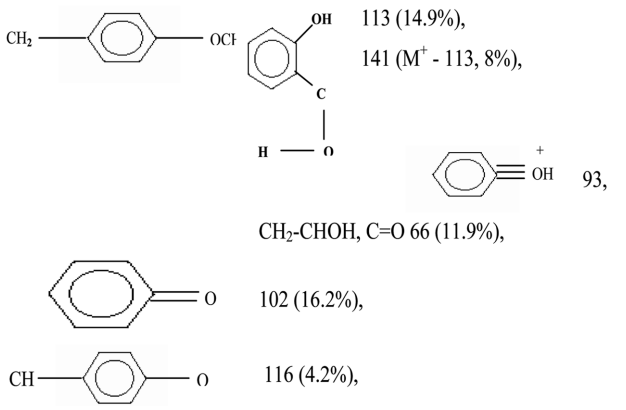


Fig. 13. Retro-Diels-Alder fragmentation mechanism of flavone.

identifies as metabolite (1) and metabolite (2).

Formation of Metabolite (1): Biotransformation of flavone by *A. niger* isolated from *Allium sativum* gave metabolite (1).

Infrared spectrum of metabolite (1) showed absorption band at 3665 Cm^{-1} indicating the presence of hydroxyl group (s) (Fig. 2). The identification of this product was based on chemical ionization mass spectrometry (Fig. 5), which indicated that M^+ at 272 (53.4%), C_4H_3 base peak 51 (100%), M^+-(C=O), 244 (13.1%).



^{13}C-NMR (off resonance and completely decoupled) showed methyl (67-97) and carbonyl (195) beside for hydroxyl groups (163.33) (Fig. 9). ^{1}H-NMR showed the presence of methyl group at 4.86 ppm (Fig. 8). The presence of aromatic hydroxyl group at (8.37 ppm) was detected. ^{1}H-NMR showed aliphatic OH at 3.28 ppm. This metabolite is produced by most microbial isolates at the early stage of fermentation, which might suggest an intermediate towards formation of the metabolite (2).

Formation of Metabolite (2): *A. niger* isolated from *Allium Sativum* was found to transform flavone to metabolite (2) with percentage increasing rate of transformation reaching up to more than 75%.

Several microbes such as *Rhizobia*, *Agrobacterium*. *Pseudomonas*, *Bacillus*, and *Rhodococcus* spp. that exist in the rhizosphere are known to participate in the breakdown or degradation of flavonoids (Barz, 1970; Barz *et al.*, 1970). Although some mechanisms have been proposed, the pathways were not elucidated. A survey of flavonoid-degrading rhizobial strains revealed that flavonoids were generally cleaved via c-ring fission (Rao *et al.*, 1991; Rao and Cooper, 1994). An early study on flavonoid degradation by a soil *Pseudomonads* indicated the presence of oxygenase based on the oxidation products and proposed that the degradation proceeds via protocatechuate production (Shultz *et al.*, 1974). The anaerobic degradation of flavonoids by the intestinal microflora, in comparison to the aerobic pathways, has been well documented, and several reports describe the reduction and dehydroxylation reactions leading to phloroglucinol formation (Gorny and Schink, 1994; Schneider *et al.*, 1999; Schneider and Blaut, 2000).

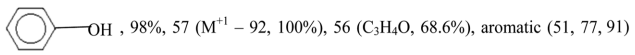
Gajendiran and Mahadevan (1988) demonstrated that *Rhizobium* could degrade the flavan-3-ol catechin, a component of condensed tannin in tannery effluent, but there are no reports of *Rhizobia* cleaving the flavone-type ring structure. Rao *et al.* (1991) reported biotransformation of the pentahydroxy flavone quercetin by *Rhizobium loti* and *Bradyrhizobium* strains. Structural characteristics suggest that protocatechuic acid originated from the B-ring of quercetin, while phloroglucinol was derived from the A-ring. Some intestinal *Clostridia* and a *Eubacterium* species can also degrade quercetin via c-ring cleavage (Krumholtz and Bryant, 1986; Winter *et al.*, 1989), but the end products are not the same, suggesting that both the pattern of ring fission and the ensuing metabolic pathways are different from those in rhizobia.

Flavone degradation has also been reported in *Pseudomonas* species (Schultz *et al.*, 1974) but with fission initiating in the A-ring via hydroxylation at C-8. Other bacterial transformations of quercetin include sulfation (Koizumi *et al.*, 1990) and glucosidation (Rao and Weisner, 1981).

Metabolite (2) infrared spectrum showed a broad band at 3639 cm^{-1} indicating a hydroxylated metabolite and another band at 873 cm^{-1} for ortho disubstituted benzene (Fig. 12).

High resolution electron impact mass spectrometry (Fig. 11) showed a molecular ion at (149, 56.9%) (M^+)

analyzed for $C_9H_9O_2$ as well as fragment ions at 92, 98%, 57 (M^{+1} – 92, 100%), 56 (C_3H_4O, 68.6%), aromatic (51, 77, 91).



^{13}C-NMR showed signals for hydroxyl group at 163.88 and carbonyl group at 205 (Fig. 9). ^{1}H-NMR also showed hydroxyl group at (8.37) (Fig. 8).

From this study it can be concluded that some of isolated fungi have the ability to transform flavone and producing more active pharmaceutical compounds. *A. niger* isolated from *Allium sativum* transformed flavone to produce two active metabolites. Flavone transformation by *A. niger* required shaking culture fermentation containing glucose as carbon source (20 gm/*l*), yeast-extract/peptone as nitrogen source (15 gm/*l*) at pH = 6.5 before autoclaving.

Flavone subjected to transformation by *A. niger* isolated from *Allium sativum*. Biotransformation process started with oxidation in the early stage with addition of oxygen atom, which is unstable. Therefore biodegradation of flavone at C1 occurred, hydroxylated, and then methylated to form metabolite (1). Biodegradation of flavone then occur where metabolite (1) was catabolized with a series of oxygenation to form transformation metabolite (2) (Fig. 14 and 15).

Biological activity of flavone and metabolites. Antimicrobial activity of flavone transformation products was studied (Table 4) against different nine microorganisms, including bacteria, fungi and yeast. Antimicrobial activity of the two products was detected using inhibition zone method and minimum inhibitory concentration (MIC)

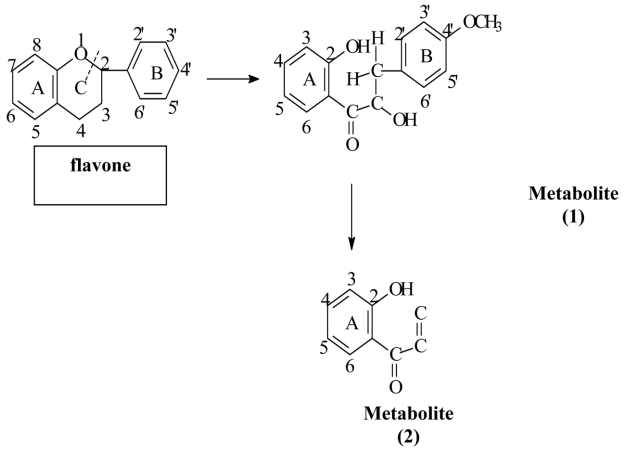


Fig. 15. Biotransformation metabolites of flavone by *A. niger*.

method (Weidenborner and Jha, 1997). Metabolite (1) and (2) had antimicrobial activity more than the starter flavone and this due to the transformation reactions added some active groups make the products more polar (more active) than flavone.

Antioxidant activity of the transformation products was studied (Table 5). The experiment was carried out using an improved ABTS decolourisation assay (Re *et al.*, 1999). Antioxidant activity was detected for both metabolite (1) and (2) which detected about 64.58% (79.5% of ascorbic acid activity), 54.16% (66.7% of ascorbic acid activity) at that order while the antioxidant activity of flavone was 37.5% (46.20% of ascorbic acid activity). These results support that antioxidant activities of flavone was increased when subjected to microbial transformation, which increased the double bondes with active groups (hydroxyl groups) which made scavenging of free-radi-

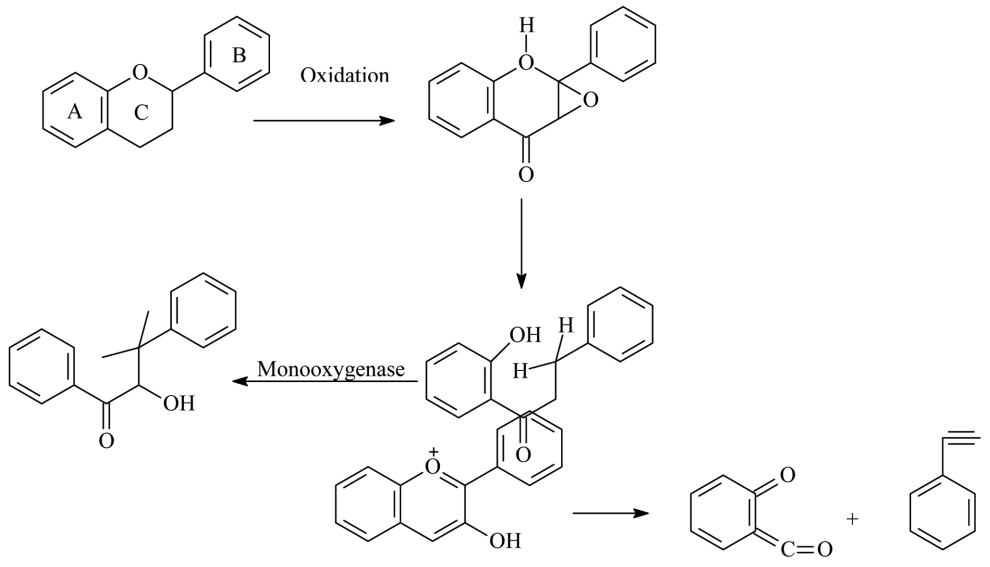


Fig. 14. Proposal mechanism of microbial degradation of flavone by *A. niger*.

cals. The absence of this double bond significantly reduces the antioxidant activity of the flavones. Salah *et al.* (1995) illustrate that, epicatechin, which also lacks this double bond, has an antioxidant activity which is only 53% of quercetin's. Epicatechin can increase its antioxidant activity with the addition of another hydroxyl group on the B ring (forming epigallocatechin), and further with the addition of gallic acid (with its three hydroxyl groups, forming epigallocatechin gallate) on the C ring, to the point where it is equivalent to quercetin.

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