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Fungal Screening of Some Natural Bioactive Purine-based Alkaloids For Efficient Biotransformation

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ABSTRACT

The utilization of naturally occurring compounds as a basis for the development of promising therapeutically active agents still attracts great interest. Unlike chemical synthesis, biotransformation provides a powerful tool for introducing complex multi-step structural modifications of substrates in a simple, cost-effective manner with mild conditions. Methylxanthines and methylurates are the most abundant, consumed, and biologically active purine alkaloids in nature. The microbial transformation of some natural purine alkaloids was explored using a collection of different filamentous fungi. Five substrates were selected for microbial screening, namely caffeine (CF), theophylline (TP), theobromine (TB), theacrine (TC), and methylliberine (ML). Results revealed the capacity of a number of fungi of different genera and species to either broadly or selectively utilize substrates. Moreover, some of these fungi could efficiently ferment the substrates and produce metabolites in a relatively high yield. These metabolites may have the potential to serve as bioactive agents that can be obtained through natural eco-friendly biotransformation.

Keywords: Microbial transformation; Caffeine; Theobromine; Theophylline; Theacrine; Methylliberine; High-efficiency fermentation; Two-stage Fermentation Bioscreening.

1. INTRODUCTION

For centuries, natural products found in plants have been recognized for their therapeutic benefits in treating various disorders, starting from common headaches to more serious conditions such as cancer and infectious diseases.[1,2] Despite the progress in drug discovery, phytomedicines are still currently used to manage many pathological conditions or act as alternative medicines. Given their chemical structure diversity, natural product-based or plant-based compounds offer an opportunity to act as potential drug leads for synthetic derivatives and structural motifs. This led to the discovery of new compounds with various therapeutic potentials.[3,4]

Alkaloids constitute around 20% of the known secondary metabolites found in plants.[5] They comprise a crucial class of natural plant products with a vast array of biological activities and effects ranging from significant therapeutic activity to being highly toxic.[6,7] Plants biosynthesize alkaloids mainly from amino acids, which result in various chemical structures.

Purine is the most abundant heterocyclic nucleus found in nature.[8] Purine alkaloids, the most recognized secondary metabolites biosynthesized from nucleotides, are widely distributed within a variety of dicotyledonous plant species such as leaves and seeds of tea (*Camellia sinensis*), coffee (*Coffea arabica*), seeds of cacao (*Theobroma cacao*) and seeds of Cola (*Cola acuminata and Cola nitida*),[9,10] Purine alkaloids exist naturally in the form of methylxanthines and

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methyluric acids, which are secondary plant metabolites originating from purine nucleotides. The structure consists of two fused six-membered pyrimidine and five-membered imidazole ring system. Naturally occurring purine alkaloids are based on xanthine (2,6-imidazopyrimidinedione) or uric acid (2,6,8- imidazopyrimidinetrione) skeletons. Methyl groups are linked to nitrogen atoms at positions 1, 3, 7, and 9 (**Table 1 and Figure 1**).[11]

Table 1. Natural purine alkaloids and different substitutions

	Purine Alkaloids	Trivial Name	R_1	R_3	R_7
1	Xanthine		H	H	H
2	7-methylxanthine	Heteroxanthine (HX)	H	H	Me
3	1,3-dimethylxanthine	Theophylline (TP)	Me	Me	H
4	3,7-dimethylxanthine	Theobromine (TB)	H	Me	Me
5	1,7-dimethylxanthine	Paraxanthine (PX)	Me	H	Me
6	1,3,7-trimethylxanthine	Caffeine (CF)	Me	Me	Me
7	1,3,7,9-tetramethyluric acid	Theacrine (TC)	-	-	-
8	*O*(2),1,9-trimethyluric acid	Liberine (LB)	-	-	H
9	*O*(2),1,7,9-tetramethyluric acid	Methylliberine (ML)	-	-	Me

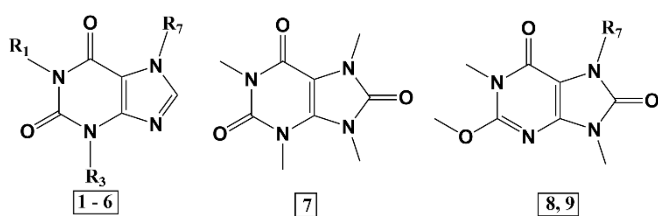

Figure 1. Chemical structures of purine alkaloids

These natural alkaloids have demonstrated a wide range of biological actions including anti-oxidant,[12,13] anti-inflammatory,[14,15] cytotoxic[16–18] in addition to beneficial systemic effects such as CNS, respiratory, cardiac, skeletal muscle stimulation, coronary dilatation, diuresis, and smooth muscle relaxation.[19] The versatile pharmacological activities of purine alkaloids were largely mediated by four different mechanisms on molecular targets at the cellular level: adenosine receptor antagonism, phosphodiesterase inhibition, GABA receptor modulation, and intracellular calcium levels regulation.[20–22]

Apart from caffeine, many methylxanthines are currently produced through chemical synthesis, which uses many chemicals and conveys numerous reactions of tedious reaction conditions, making it an expensive, environmentally hazardous, and complex process. Establishing a microbial platform for large-scale production of these compounds could provide a simple, economical, and environmentally friendly approach.[23]

Biotransformation is a metabolic process involving the introduction of specific modifications to natural or synthetic compounds resulting in structurally similar less toxic derivatives. Different biological catalysts can be used to perform this process, such as bacteria and fungi with their unique enzymes.[24,25] Biotransformation is gaining popularity as an alternative to traditional chemical catalysis, using biocatalysts to improve yields and simplify bioprocessing under environmentally safe conditions.[26] Moreover, it has the potential to establish the groundwork for entirely new products and replace conventional chemical production methods.[27]

Microorganisms have been proven to metabolize substrates in a way that mimics mammals, thus serving as a model for mammalian metabolism. Pathways of mammalian metabolism include functionalization reactions (phase I) such as oxidation with cytochrome P450, reduction, and hydrolysis, and phase II reactions such as glucuronidation, methylation, acetylation, and sulphation.[28,29]

Under specific nutritional conditions, microorganisms can produce a large number of enzymes that are difficult to be produced using traditional synthetic methods. They can also produce various products.[27] Biocatalysis simplifies drug synthesis by eliminating the need for complex separation and purification steps and selectively producing target products under mild fermentation conditions.[24] The most significant aspect of biotransformation is that it preserves the original carbon skeleton while obtaining products with minimal waste production and energy consumption. Microbial transformation also shows high specificity, enantioselectivity, and regioselectivity.[30]

Although purine alkaloids are toxic to many microorganisms and invertebrates, some organisms have evolved to use them as carbon and nitrogen sources.[31,32] Therefore, it is not uncommon to find fungal and bacterial strains that are resistant to purine alkaloids.[33] Multitudes of researchers have shown keen interest in investigating their metabolism using different organisms.[23,34–36] Despite the metabolism of methylxanthines in bacteria has been intensely studied for many decades,[37,38]. There have been few studies on their fungal degradation, with a few numbers of fungi being isolated and identified that were capable of metabolizing methylxanthines, including *Aspergillus*, *Rhizopus*,[39] *Penicillium*,[40] *Fusarium*,[41] *Chrysosporium,* and *Gliocladium*.[42] To our knowledge, there have been no previous reports of any biotransformation studies carried out on methylurates (TC and ML).

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Due to the broad range of biological activities displayed by purine alkaloids, advantages of microbial transformation, little fungal transformation studies on methylxanthines, and lack of biotransformation studies on methylurates, this screening study was conducted using a collection of microorganisms hoping to shed some light on this foremost subject.

2. RESULTS AND DISCUSSION

The principal goal of this screening study was to detect the most relatively efficient microorganisms for the production of metabolites. These fungi can be further employed in a large-scale fermentation study for isolation and purification of purine metabolites and assessment of their biological activities. The summary of the results is illustrated in (**Table 2**). The efficiency of metabolism was determined based on the relative intensities of TLC spots and was assigned as no conversion (-) for the absence of spots, trace conversion (+) for one or more low-intensity spots, moderate conversion (++) for one or more medium intensity spots and high conversion (+++) for at least one high-intensity spot. The results showed that out of the 42 tested microorganisms (**Table 3**), 14 of them were able to metabolize at least one of the substrates with variable efficiencies without optimization. Meanwhile, the rest of the screened fungi were unable to metabolize any of the five substrates.

Table 2. Results of substrates' screening for efficient microbial transformation with number of spots (NOS) and optimum conversion time (OCT).

Microorganism	CF		TP		TB		TC		ML	
	NOS	OCT	NOS	OCT	NOS	OCT	NOS	OCT	NOS	OCT
Alternaria alternata AUMC 4685	+		–		–		–		–	
	3	10,14								
Aspergillus flavus AUMC 4787	–		–		+				+	
					2	7,10			2	14
Aspergillus niger NRRL 328	++		++		–		+++		+++	
	2	10	1	10			2	14	2	10
Aspergillus niger ATCC 10549	+		–		+		–		–	
	1	7			1	14				
Aspergillus versicolor AUMC 4807	+++		–		++		–		–	
	2	7,14			1	10				
Cunninghamella blackesleeana NRRL 1369	–		–		–		–		+++	
									1	7
Cunninghamella echinulata NRRL 1382	+++		+		++		+		–	
	1	14	1	14	1	10	1	7		
Cunninghamella elegans NRRL 1392	–		–		–		+		–	
							2	10		
Cunninghamella elegans NRRL 2310	–		–		–		–		+	
									1	10
Penicillium brevicompactum AUMC 2751	+		–		–		–		–	
	2	14								
Penicillum chrysogenum ATCC 9480	++		–		+		+++		++	
	1	7			1	7	2	14	1	10
Penicillium vermiculatum NRRL 1009	+		+		–		–		–	
	1	3	1	7						
Rhodotorula rubra NRRL 1592	–		+		–		–		–	
			1	3						
Rhizobus somniferum ATCC 36060	+		–		–		+		–	
	2	10					2	14		

- No conversion of substrates
+ Trace conversion of substrates into metabolites.
++ Moderate conversion of substrates into metabolites.
+++ High conversion of substrates into metabolites without optimization.

Analysis of screening results showed that *Alternaria alternata* AUMC 4685, *C. blackesleeana* NRRL 1369, *C. elegans* NRRL 1392, NRRL 2310, *P. brevicompactum* AUMC 2751, and *R. rubra* NRRL 1592 could metabolize only one of the five purine substrates. This finding suggested that these aforementioned fungi had a high degree of substrate selectivity. On the other hand, other fungi such as *A. niger* NRRL 328, *C. echinulata* NRRL 1382, and *P. chrysogenum* ATCC 9480 exhibited broad selectivity for purine substrates as each one of them could metabolize four of the five substrates with various efficiencies. Finally, *A. flavus* AUMC 4787, *A. niger* ATCC 10549, *A. versicolor* AUMC 4807, *P. vermiculatum* NRRL 1009, and *R. somniferum* ATCC 36060 showed some degree of metabolic selectivity being able to ferment two substrates.

Regarding the metabolic efficiency of the screened fungi, they showed a wide spectrum from no trace conversion of substrates to relatively high yield conversion with a variable number of metabolites towards specific substrates. *A. niger* NRRL 328 showed high metabolic efficiency against TC and ML and moderate efficiency towards CF and TP. *A. versicolor* AUMC 4807 was highly efficient in metabolizing CF and moderate towards TB, while *C. blackesleeana* NRRL 1369 was able to metabolize ML with great efficiency. Additionally, *C. echinulata* NRRL 1382 showed a high yield conversion of CF and moderate conversion of TB. Lastly, TC was efficiently metabolized by *P. chrysogenum* ATCC 9480, whereas CF and ML were moderately converted to metabolites by the same fungus.

Some of the screened fungi gave rise to more than one metabolite, with a few of them producing these metabolites at different conversion times. In other words, one metabolite was formed at a certain time and then started to fade away as another metabolite was formed, suggesting sequential metabolism and utilization of metabolites as intermediates. Such fungi were *A. alternata* AUMC 4685 and *A. versicolor* AUMC 4807 with CF, and *A. flavus* AUMC 4787 with TB.

Addressing the chemical properties of the formed metabolites, most of these metabolites were more polar than their respective substrates. Interestingly, the second metabolite (14th day) produced by *A. versicolor* AUMC 4807 against CF and the one against TB together with the metabolite resulting from the transformation of TP by *R. rubra* NRRL 1592 were less polar than their respective substrates.

3. EXPERIMENTAL TECHNIQUES

3.1. General Experimental Procedure

TLC was carried out using precoated TLC sheets silica gel G60 F_{254} sheets (E. Merck, Germany). All reagents and solvents used for separation and purification were of analytical grade. A UV lamp ($\lambda = 254$ and 365 nm) was used for visualization of the TLC plates. All chemicals used were purchased from Sigma–Aldrich (St. Louis, Mo, USA). Eight solvent systems were utilized for analysis of TLC; SS_1: methylene chloride-methanol (85:15, v/v); SS_2: ethyl acetate-methanol (95:5, v/v); SS_3: ethyl acetate-methanol-ammonia (95:5:5, v/v); SS_4: methylene chloride-methanol (95:5, v/v); SS_5: methylene chloride-methanol (75:25, v/v); SS_6: methylene chloride-methanol-ammonia (95:5:5, v/v); SS_7: ethyl acetate-methanol (98:2, v/v); SS_8: methylene chloride-methanol (90:10, v/v).

3.2. Chemicals

Caffeine, theophylline, and theobromine were obtained from Sigma Aldrich Chemical Company Inc, USA. Theacrine and methylliberine were obtained from Bulk Stimulants Company Inc. (Florida, USA). Purity was confirmed using TLC. Substrates were added to stage II culture as a solution in warm sterile water (10 mg / 0.5 ml solution using vortex apparatus inoculated in 250 ml Erlenmeyer flask containing 50 ml stage II culture media).

Materials for preparation of biotransformation media were as follows: sabouraud-dextrose agar, SDA (Becton Dickinson and Co., Cockeysville, Med 21030), yeast extract microbiological grade (Oxoid LTD, 329185, England), peptone microbiological grade (Sigma Chemical Co., St. Louise, Mo., 63118, USA), potato- dextrose agar PDA (DIFCO, Detroit, 48201, USA), dextrose AR-grade (Sigma Chemical Co., St. Louise, Mo., 63118, USA), sodium chloride, analytical grade, dipotassium hydrogen phosphate (ADWIC Co), glycerin (ADWIC Co) and nutrient agar (Oxoid LTD, 329185, England).

3.3. Microorganisms

Forty-two microbial cultures were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), Northern Regional Research Laboratories (NRRL, Peoria, Illinois, USA), and Assiut University Mycological Center (AUMC, Assuit, Egypt). A list of these microorganisms is shown in **Table (3)**.

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Table 3. List of screened fungi.

No.	Fungus	Code
1	*A. alternata*	AUMC 4685
2	*Aspergillus alliaceous*	NRRL 315
3	*Aspergillus corymbiform*	**
4	*Aspergillus flavipes*	ATCC 11013
5	*A. flavus*	AUMC 4787
6	*Aspergillus fumigates*	**
7	*A. niger*	NRRL 599
8	*A. niger*	NRRL 2295
9	*A. niger*	ATCC 10549
10	*A. niger*	**
11	*A. niger*	NRRL 328
12	*Aspergillus ochraceous*	NRRL 398
13	*Aspergillus ochraceous*	NRRL 405
14	*A. versicolor*	AUMC 4807
15	*Candida albicans* (Lab isolate)	*
16	*Chaetomium funiculum*	**
17	*Chimaphila umbellate*	**
18	*Cladosporium* species	**
19	*Cochliobolus* species	**
20	*C. blackesleeana*	NRRL 198
21	*C. blackesleeana*	NRRL 1369
22	*C. echinulata*	NRRL 1382
23	*C. elegans*	NRRL 1392
24	*C. elegans*	NRRL 2310
25	*Dreshella* Species	**
26	*Fusarium solani*	**
27	*Gymnascella citrina*	NRRL 6050
28	*Lindera pinnespora*	NRRL 2237
29	*Mucor* species	**
30	*Paecibomyces* species	**
31	*Penicillium brevicompactum*	AUMC 2751
32	*Penicillium chrysogeneum*	ATCC 10002
33	*Penicillium chrysogeneum*	ATCC 9480
34	*Penicillium duclauxii*	**
35	*Penicillium glabrum*	**
36	*Penicillium vermiculatum*	NRRL 1009
37	*Rhizopus somniferum*	ATCC 36060
38	*Rhodotorula rubra*	NRRL 1592
39	*Saccharomyces cerivisae* (Baker`s yeast)	Lyophilized yeast cell
40	*Stachybotrys* species	**
41	T*richoderma viride*	**
42	*Ulocladium botrytis*	**

*Laboratory isolate supplied through the Department of Microbiology, College Pharmacy, King Saud University.

**Culture collection obtained from Assiut University Mycological Center (AUMC).

Solid media (slants) were prepared by dissolving potato dextrose agar (11 g) or sabouraud-dextrose agar (18 g) and nutrient agar (3 g) in distilled water (300 ml) and sterilized by autoclave at 121°C and 15 psi for 15 min. The cultures were started by rehydrating the lyophilized cells with 1 ml of sterile water. To check for purity, a small amount of suspended cells was streaked on sabouraud-dextrose agar plates. Pure cultures were kept on slants of either potato-dextrose agar or sabouraud-dextrose agar. The fresh slants were left to sit at room temperature for a few hours before being stored in a refrigerator at 4°C and subcultured every three months.[43]

3.4. Microbial Screening

A preliminary screening procedure was conducted using a two-stage fermentation protocol (**Figure 2**).[43] Stage I cultures were initiated from two-week-old slants (under aseptic conditions) by transferring microbial cells into a 250 ml Erlenmeyer flask containing 50 ml of sterile liquid medium and allowed to grow for 72 hr at 27°C on a gyratory shaker operating at 150 rpm. The liquid medium used for the cultures was composed of 20 g of dextrose, 5 g of yeast extract, 5 g of K_2HPO_4, 5 g of peptone, and 5 g of NaCl per liter of distilled water, with the pH adjusted to 7.0 before autoclaving at 121°C and 15 psi for 15 minutes.

Stage II cultures were obtained by transferring 5 ml of stage I culture to each of the 250 ml flasks containing 50 ml of fresh liquid medium. Both substrate and organism controls were treated in the same way. Cultures were allowed to grow for 24 hr before the addition of the substrate dissolved in warm sterile water using a vortex apparatus. The solutions were added to each flask separately in the form of 0.5 ml containing 10 mg of each substrate. Samples of the liquid culture, 5 ml each, were periodically withdrawn from each culture on the 3rd, 7th, 10^{th,} and 14th day of fermentation and extracted successively with chloroform, ethyl acetate, and n-butanol. Each extract was separately evaporated to dryness under reduced pressure and kept for TLC examination. Control cultures were grown under the same conditions but without adding any substrate. Substrate controls consisted of a sterile medium to which the substrate was added and then incubated without microorganisms.

The obtained residues were dissolved in a minimal amount of suitable solvent and examined by thin-layer chromatography on precoated silica gel G60 TLC sheets using solvent systems SS_1 - SS_8. The plates were dried and visualized under UV light at a wavelength (λ) of 254 and 365 nm.

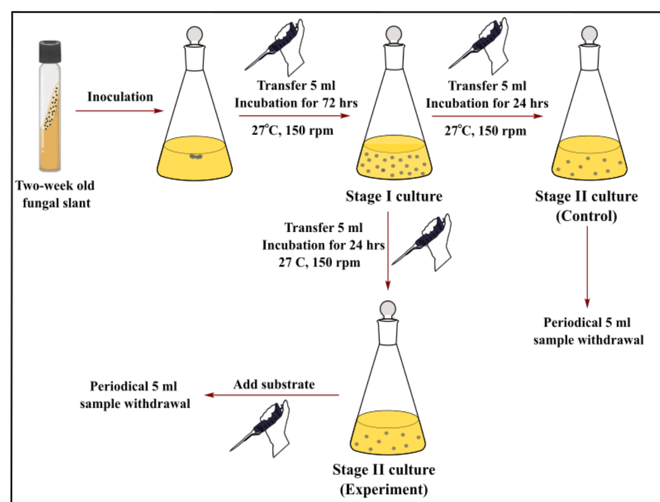

Figure 2. The screening process of substrates with 42 Fungi using a two-stage fermentation protocol.

4. CONCLUSION

This fermentation study revealed that some of the screened fungi could metabolize one or more of the purine alkaloids' substrates with varying degrees of selectivity and efficiency. This could be implemented in a large-scale fermentation study to isolate and identify the metabolites in addition to evaluating their possible therapeutic effects. This study could also serve as a starting point to explore the different enzymatic reactions and possible regioselectivity relative to the purine nucleus. It could provide a means to establish parallelism between the fungal, bacterial, and mammalian metabolism of purine alkaloids.

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