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Cytotoxic effects of Mannich bases via induction of caspase-3 pathway on human oral squamous cell carcinoma

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Abstract: In the anticancer drug research, there is a need for the synthesis of compounds with selective cytotoxicity compared to the anticancer drugs in the market. The current study aimed to determine the cytotoxicities of the bis Mannich bases **1-9** towards human oral squamous cell carcinoma (OSCC). Mannich bases showed promising cytotoxicity in low micromolar in the range of 1.7-27 μM against OSCC cell lines. The compounds **5** with the highest potency selectivity expression (PSE) value (318.1) and **7** with the highest tumor selectivity (TS) values (TS1:11.2, TS2:15.8) showed promising selective cytotoxicity towards cancer cell lines. Furthemore, Western blot analysis showed that the representative compound **7** induced the activation of caspase-3 in HSC-2 cells. These results may suggest that the apoptotic pathway may be one of the possible mechanisms of the action and the lead compound **7** can be subjected to the further bioassays and molecular design.

Keywords: Mannich base, anticancer, apoptosis, PARP

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INTRODUCTION

World Health Organization (WHO) cancer reports indicate that cancer is a principal health issue that causes deaths worldwide after cardiovascular system diseases. The most common types of cancer in men are lung, prostate, and colorectum, while those are the breast, colorectum, and lung in women (1). Among cancer types, oral cancers are part of head and neck cancers. According to the American Cancer Society, in 2020, 10.750 people will die from oral cancer in the US. It is indicated that tobacco, alcohol consumption, and several viral infections are primary risk factors (2). The main drawbacks of current chemotherapeutic drugs are known as selectivity deficiency, severe side effects, and multidrug resistance (3). Therefore, new and promising anticancer drug candidates for clinical trials is needed.

Mannich reaction is a useful way to obtain aminoalkylated compounds which are used as prodrugs in medicinal chemistry studies. Aminoalkylation of the compounds mostly affects the lipophilicity of the molecule, pKa, and absorption process through membranes (4). Besides, Mannich bases turn into α, β- unsaturated ketone moiety under suitable conditions and they act as thiol alkylators in cancer cells. This situation may provide advantages compared to available anticancer drugs since during the cell division thiol bearing glutathione levels were increased. Therefore, α, β-unsaturated ketones interact with the thiol group, except hydroxy and amine moieties that are available in proteins. Also, they may potentially show fewer side effects and selective cytotoxicity towards cancer cells except for normal cells (5-9). Large numbers of Mannich bases in different chemical structures such as ketonic, phenolic, and alkyne type compounds, etc were reported with anticonvulsant, analgesic,

antifungal, anticancer and antioxidant activities (4).

Chalcones having the structure of 1,3-diaryl-2-propen-1-one is one of the most privileged scaffolds in medicinal chemistry since they have a functional chemical skeleton to design many kinds of compounds, and they have also been reported with valuable bioactivities such as anticancer, anti-diabetic, antioxidant, anti-inflammatory, and anti-infective effects (10). Conversion of phenolic chalcones into the related Mannich bases generally increases their cytotoxic effects (6, 11-16). This promising behavior may be a result of additional alkylation centers formed by the chalcone to lead to excessive cytotoxic effects by interaction with more cellular thiols based on the sequential cytotoxicity hypothesis (5, 6, 17).

Mannich reaction as a powerful tool in medicinal chemistry is considered both for the synthesis of drug candidates and for the modification of physicochemical properties of the compound to direct its pharmaco-kinetic properties. In this study, cytotoxic/anticancer properties of Mannich bases, 1-(3,5-bis-aminomethyl-4-hydroxyphenyl)-3-(4-substituted phenyl)-2-propen-1-ones **1**-**9** (Table 1), were investigated *via* MTT test against human oral squamous cell carcinoma (OSCC) cell lines and human normal oral cells. Moreover, the mechanism of action of the representative compound was investigated *via* Western blot analysis to find out how the most potent compound affects cancer cells.

EXPERIMENTAL SECTION

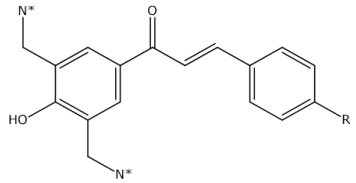
Determination of the cytotoxicities *via* MTT and Western blot analysis

Cytotoxicity assay was realized according to our previous studies (13, 18, 19). Human oral squamous cell carcinoma cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) and human normal oral cells (HGF, HPLF, HPC) were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay test in this study. Reference drugs were Doxorubicin and 5-Fluorouracil (5-FU). The mean value of CC_{50} for each cell type was calculated from triplicate assays. The selectivity index (SI) value was calculated by the quotient of the average CC_{50} value for non-malignant cells (HGF, HPLF, HPC) divided by the CC_{50} value for a specific tumor cell line (Ca9-22, HSC-2, HSC-3 or HSC-4). Tumor selectivity (TS) values (TS1) was calculated by dividing the average CC_{50} value towards normal cells into the average CC_{50} value towards cancer cell lines. (Column D/Column B, Table 2) and TS values (TS2) were generated for a compound by dividing the average CC_{50} value towards HGF cells into the CC_{50} value towards Ca9-22 cells (Column C/Column A, Table 2). A potency selectivity expression (PSE) values were calculated by dividing average CC_{50} values towards OSCC cell lines (a measure of potency) and the average SI figures towards these cell lines (a determination of tumor-selectivity) and expressed as a percentage. Western blot analysis was realized as described previously(20).

RESULTS AND DISCUSSION

The synthesis method of the compounds 1-9 (Table 1) was reported previously by our group (21). Target compounds 1-9 were obtained by the reaction of a suitable chalcone, paraformaldehyde, and suitable secondary amines such as piperidine (1, 4, 7) morpholine (2, 5, 8), and *N*-methyl piperazine (3, 6, 9) under microwave conditions and the chemical structures of the compounds were verified by ^{1}H NMR, ^{13}C NMR, and HRMS (21).

Table 1. General chemical structure of the Mannich bases **1**-**9**.



Compound	R group	N* group
1	-CH_3	Piperidine
2		Morpholine
3		*N*-methyl piperazine
4	-OCH_3	Piperidine
5		Morpholine
6		*N*-methyl piperazine
7	-NO_2	Piperidine
8		Morpholine
9		*N*-methyl piperazine

Cytotoxicities of the compounds **1**-**9** were presented in Table 2. The compounds showed remarkable cytotoxicities in the low micromolar concentration range of 1.7-27 µM against cancer cell lines. The compounds generally more powerful cytotoxic agents than 5-FU, even so, they were less effective compared to Doxorubicin. All compounds showed cytotoxic effect with the lowest average CC_{50} values of 2.5-12.9 µM against OSCC cell lines than reference drug 5-FU (16.9 µM), except the compounds **4** and **5**. When the results were considered, compound **7** (9.6 times, on Ca9-22), and compound **9** (7.7 times, on HSC-2; 6 times on HSC-3; 5.7 times on HSC-4) were found more cytotoxic compounds among others than 5-FU against the cell issued.

Selectivity index (SI), which is greater than the value of 1, indicates that the compound tested has selective cytotoxicity towards cancer cells, and that can be forwarded to further investigations (17, 22). Calculated SI values towards OSCC cell lines were in the range of 2.7-16.7. It seems that all compounds have selective cytotoxicity towards cancer cells. In addition, according to the average SI values (4.7-12.2) against OSCC cell lines, compound 7 drawn great attention, with the highest average SI value (12.2) against OSCC cell lines as a lead compound.

Tumor selectivity (TS) values (TS1 and TS2, Table 2) were calculated in two ways. According to TS1 values, compound 7 had the highest TS value (11.2), among others. HGF cells and Ca9-22 cell lines were produced from the same origin. Therefore, the calculation of TS2 values was done to understand tumor selectivity in terms of different aspects. The compounds 1, 2, and 7 had the highest TS2 values of 12.6, 13.6, and 15.8, respectively. Two TS calculations indicated that compound 7 was a tumor-selective compound in series.

The lead compound, which has selective toxicity towards cancer cells, was determined according to potency selectivity expression (PSE) values in Table 2. PSE values were calculated in the range of 28-318.1. The compounds **1**-**3** having 4-methylphenyl and the compounds **4**-**6** having 4-methoxyphenyl had higher PSE values (197.5, 119.4, 118.8, 264.7, 318.1, 161.4, respectively) than **7**-**9** having 4-nitrophenyl (28, 65.1, 53.1, respectively). It can be concluded that the PSE values of the compounds prominently increased when electron-releasing groups were substituted on the phenyl ring. Furthermore, the promising lead (PL) concept (17, 23) was considered to determine the lead compound. Based on this concept, promising lead compounds have CC_{50} < 10 µM and SI >10. In this study, PL10 criteria have been achieved with the compounds **2** (CC_{50}=8.8 µM, SI=10.2) and **7** (CC_{50}=2.6 µM, SI=14.7) towards Ca9-22 and the compound 6 (CC_{50}=3.7 µM, SI=10.8) and 7 (CC_{50}=2.3 µM, SI=16.7) towards HSC-4 cell line.

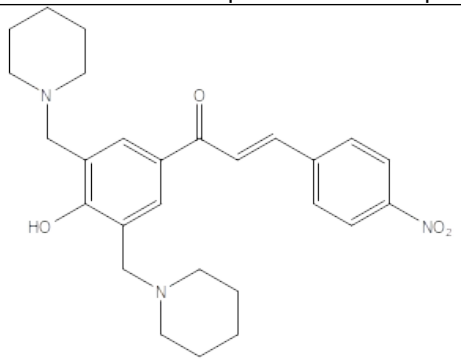
According to results and evaluations, compound **7** made attraction, and also its drug-likeness properties were theoretically predicted, as shown in Table 3 using SwissADME web tool (24). The physicochemical properties of compound **7** such as the logarithm of the partition coefficient (log P), molecular weight (MW), the number of hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), rotatable bonds (RB), and total polar surface area (TPSA) were calculated to see if the compound **7** has drug-likeness properties. According to Lipinski and Veber filters, it can be expressed that compound **7** had a drug candidate potency with its compatible values.

Table 2. Cytotoxicities of the compounds 1-9 against OSCC cell lines.

	CC_{50} (μM)																			
	Human oral squamous cell carcinoma cell lines (OSCC)											Human normal oral cells								
	Ca9-22	SI	HSC-2	SI	HSC-3	SI	HSC-4	SI	Mean	SD	Mean	HGF	HPLF	HPC	Mean	SD	TS			
Compounds	(A)								(B)		SI	(C)			(D)		(D)/(B)	(C)/(A)	PSE	logP
1	10.0	8.0	15.0	5.3	17.0	4.7	9.7	8.2	12.9	3.6	6.5	126.0	54.0	59.0	79.7	40.2	6.2	12.6	197.5	5.32
2	8.8	10.2	14.0	6.4	15.0	6.0	6.3	14.3	11.0	4.2	9.2	120.0	49.0	101.0	90.0	36.8	8.2	13.6	119.4	3.20
3	8.4	4.1	8.2	4.2	13.0	2.7	2.2	15.8	8.0	4.4	6.7	44.0	42.0	18.0	34.7	14.5	4.4	5.2	118.8	3.29
4	15.0	6.6	15.0	6.6	27.0	3.6	11.0	8.9	17.0	6.9	6.4	140.0	55.0	100.0	98.3	42.5	5.8	9.3	264.7	4.93
5	17.0	6.3	20.0	5.4	23.0	4.7	15.0	7.2	18.8	3.5	5.9	109.0	101.0	113.0	107.7	6.1	5.7	6.4	318.1	2.80
6	13.0	3.1	12.0	3.3	7.6	5.3	3.7	10.8	9.1	4.3	5.6	42.0	42.0	36.0	40.0	3.5	4.4	3.2	161.4	2.90
7	2.6	14.7	4.3	8.9	4.5	8.5	2.3	16.7	3.4	1.1	12.2	41.0	35.0	39.0	38.3	3.1	11.2	15.8	28.0	4.83
8	5.4	6.7	4.5	8.1	5.0	7.3	4.6	7.9	4.9	0.4	7.5	42.0	40.0	27.0	36.3	8.1	7.5	7.8	65.1	2.71
9	3.5	3.1	2.6	4.2	2.2	5.0	1.7	6.5	2.5	0.8	4.7	11.0	10.0	12.0	11.0	1.0	4.4	3.1	53.1	2.80
Doxorubicin	0.1	70.8	0.1	70.1	0.1	77.3	0.1	79.1	0.1	0.0	74.3	8.5	1,9	10.0	6.8	4.3	74.1	88.5	0.1	-
5-FU	25.0	38.4	20.0	48.0	13.0	73.8	9.7	98.9	16.9	6.9	64.8	1000.0	1000.0	879.0	959.7	69.9	56.7	40.0	26.1	-

Each value represents the mean ±S.D. of triplicate determinations. Human gingival fibroblast (HGF); Human periodontal ligament fibroblast (HPLF); Human pulp cell (HPC); Oral squamous cell carcinoma cell lines (OSCC: Ca9-22, HSC-2, HSC-3 and HSC-4); Tumor-selectivity index (TS); Potency-selectivity expression (PSE); Selective index (SI); 50% cytotoxic concentration (CC_{50}); SD standard deviation. logP values calculated by Molinspiration Cheminformatics online program.

Table 3. Druglikeness properties of the most potent lead compound **7**.



Compound	logP	MW	HBA	HBD	RB	TPSA
7	3,63	463,57	6	1	8	89,6
Druglike compound	<4.15	<500	<10	<5	<10	<140

Lipinski (Pfizer) filter: MW ≤ 500, logP ≤ 4.15, N or O ≤10, NH, or OH ≤5.
Veber (GSK) filter: RB≤10, TPSA≤140

A partition coefficient (P) or its logarithmic value of logP is an indicator parameter of the solubility properties of the drug candidates. Among series, bis Mannich bases **1**, **4**, and **7**, which have piperidine, had the highest logP values of 5.32, 4.93, and 4.83, respectively, in series (Table 2). As for logP, CC_{50}, SI, and TS values of compound **7** were compared with other nitro derivatives, it can be stated that the piperidine ring led to having high logP value, and this situation resulted in increased cytotoxicity of the compound **7** against OSCC cell lines. It is known that lipophilicity enhances the absorption of the compounds through the cell membrane. Therefore, the compound may easily accumulate in the cell and it may cause increased bioactivity.

Apoptosis, a programmed cell death process, has a role in regulating cell proliferation triggered by many anticancer drugs. Caspase-3 protein is known as one of the targets in the apoptotic process (25). Western blot analysis demonstrated that compound **7** induced the production of a cleaved product of PARP and the activation of caspase-3 in HSC-2 as potently as actinomycin-D, which was a positive control of apoptosis (Figure 1).

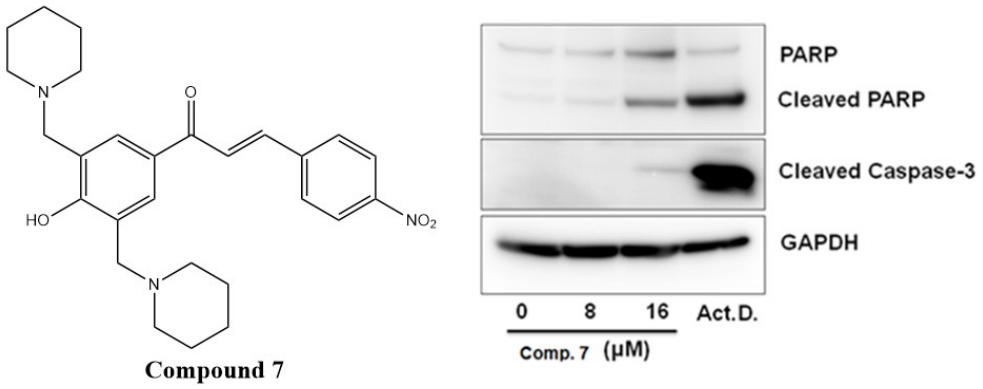


Figure 1. Western blot analysis of the representative compound **7** on HSC-2 cells.

CONCLUSION

In conclusion, this research was focused on to investigating the cytotoxic effects of Mannich bases on several cancer cell lines. The CC_{50} values of the bis Mannich bases were found in the range of 1.7-27 μM against OSCC cell lines. When tumor selectivity (TS1 and TS2) and potency selectivity expression (PSE) were considered, compound 5 with the highest PSE value (PSE=318.1) and compound 7 with the highest TS values (TS1=11.2 and TS2=15.8) made great attraction. The most selective cytotoxic compound 7 induced apoptosis process in HSC-2 cells. The compound 7 can also be subjected to further bioassays on different cancer cells for future studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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