"Science Stays True Here"
Biological and Chemical Research, Volume 3, 234-245 | Science Signpost Publishing

SSPub

Antioxidant Activity and Cytotoxicity of Nepalese Nak Cheese

Katawal I., Kharel G.P., Poudel B.H.

Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Received: April 23, 2016 / Accepted: May 21, 2015 / Published: August 25, 2016

Abstract: Nepal was the first country in Asia to make any Western-style cheeses, and until the 1980s certainly the only Asian country making *nak* Cheese (McGee, 2008). The aims of this work were to study the functional aspects of *nak* cheese produced in Nepal in respect to its antioxidant activity and cytotoxicity. Three *nak* cheeses and a cow cheese were collected randomly from regional factories of Dairy Development Corporation (DDC) in Nepal, located at altitude of 2900 m, 2600 m, 2400 m and 1900 m respectively. The cheeses were subjected to phytochemical extraction using methanol as solvent followed by quantification of the total phenol content (TPC) and flavonoid content (TFC) in their extracts. The TPC and TFC of the nak cheeses were 6.06-10.9 mg GA/g dry extract and 4.12-7.70 mg QE/g dry extract respectively which were significantly higher ($p<0.05$) than the cow cheese that gave the corresponding values of 2.75 mg GA/g dry extract and 1.80 mg QE/g dry extract respectively. Similarly, antioxidant activity (IC50) assayed using DPPH radicals and toxicity (LC50) determined using Brine shrimp lethality test, of the studied *nak* cheeses extract were 371.64-518.30 mg/L and 350.19-698.95 µg/mL (i.e., moderate toxicity) respectively. In contrast, cow cheese (1900m) gave IC50 of 626.24 mg/L, significantly lower ($P<0.05$) than the studied *nak* cheeses but its toxicity (LC50) of 376.84 µg/mL was in the range obtained for *nak* cheeses. Thus, *nak* cheese may be considered as good source of antioxidants and its regular consumption might improve consumer health due to antioxidant activity and anti-cancerous effect.

Keywords: Functional food, Nak cheese, Phytochemicals, antioxidant, cytotoxicity

---

Corresponding author:
Katawal I., Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal. Email: ishwarkatawal16@gmail.com.

1. Introduction

Female yak are called "*dri*" in Tibet; "*nak*" in Nepal and male is called yak. They are scientifically called *Bos grunniens* (Linnaeus, 1766). Yak and nak reared at higher altitude above 3,000 m. Yak is raised in 22 Himalayan districts of Nepal along the Tibetan boarders with long hairs and pointed horns. Yak and *nak* provides milk and meat for human consumption (Thapa, 1994).

Unfortunately, *nak* is unable to survive at lower altitudes and gives only 1-2 liters of milk in a day on average. Hybrid of nak called *nak*-cattle hybrid (Chauri) are produced by crossing Yak with female cow called Aulo and nak with local bull known as Kirko. The hybrid is thus able to survive at lower altitudes (upto 1800 m) and has high milk production (485 litres on average during 6 months of lactation). The hybrid milk has fat content about 5-6% whereas a pure *nak* has 7-9% milk fat (Indra, 1997). *Nak* cheese also popularly known as "Yak cheese" is produced from *nak* or *nak*-cattle hybrid. *Nak* cheese made in Nepal has half the butterfat skimmed off the milk to make a harder- style cheese and is soaked in brine and cured outdoors in shelters. The cheese ends up grainy and tangy (Thapa, 1994). Cheese is rich in dietary oligopeptides such as casein and whey protein. The presence of phytochemicals in the milk and later in the cheese is a result of their transfer from plant to milk (McIntosh et al, 1995). They can influence milk and cheese taste and can also affect their antioxidant activity. Geographical location determines the content of phytochemicals (antioxidants) in plant (Taylor et al., 1997) and antioxidants peptides in raw milk of animals (Alyaqoubi et al, 2015). Recent research have claimed that Grass feeding improves the quality of the cheese and makes the cheese richer in omega-3 fats, vitamin E, and CLA (a beneficial fatty acid named "conjugated linoleic acid"). "Grass- feeding also increases the amount of another key antioxidant—beta-carotene—in cow's milk about 4 times higher than the level in milk from conventionally fed cows. (Coppa et al, 2011 and Hilario et al, 2010). Ripening period of cheese (Yasuda et al, 2012) and its microbiology (Sosa et al, 2001) as well as breed (Alyaqoubi et al, 2015) of the animals are other factors that play an important role in the concentration of antioxidant in cheese. On such context Yaks and *naks* are acclimatized to cold climate of higher altitudes, have different feeding habits, forage on variety of flora and fauna of the Alpine Pasture and feed on rare and medicinally important herbs. Most importantly Nak are 100% grass fed, and differ genetically from cow. Similarly ripening period of *nak* cheese and its microbiology also differs from cow cheese. So the *Nak* cheese could contain higher or significantly different content of phytochemicals and antioxidants compared to cow cheese. The presence of phytochemicals and antioxidant peptides could deliver significant antioxidant potential to nak cheese.

Nak cheese has become an important part of the regular diet of people at higher altitudes and being consumed by most of the Nepalese. Numerous basic studies have demonstrated that a part of dairy and other plant-originated natural food products may possess anticancer activity. Researchers have found that cheese extract showed lower cytotoxic effect in differentiated HL-60 cells than undifferentiated HL-60 cells at the varying concentrations tested (Yasuda et al, 2012). On such context nak cheese could have the presence of biological components and peptides with anti-cancerous potential. Researchers such as Yasuda et al, 2012 have shown the strong influence of cheese ripening duration to its cytotoxicity, therefore nak cheese that are being ripened over 3 months could exhibit significant cytotoxicity, which needs to be evaluated.

2. Materials and Methods

Sample Collection

Four types of Commercial cheese including one cow cheese known as Kanchan cheese from 1900 m, and three nak cheese were sampled from cheese factories of Dairy Development Corporation. Nak cheeses were collected from 2900 m, 2600 m and 2400 m respectively. The samples were of varying ripening period with cow cheese ripened for a month and nak cheese ripened over three months.

3. Phytochemical Analysis of Cheese

Preparation of cheese extract

In order to perform phytochemical analysis, aseptically sliced cheese were grinded and extraction solvent methanol (Thermo-Fisher, India) was added (750ml per 100g of dry sample). Sonication was carried out for three days. Then the extracts were filtered under vacuum and concentrated at reduced pressure using a rotary evaporator at 37 ^{o}C. The dried extracts were kept in the refrigerator at 4 $^{\circ}$C until use (Sethi et al, 2013).

Determination of total phenol content

Total Phenolic Content was determined by using Folin- Ciocalteu method with Gallic acid (Molychem) as standard as described by Gao (2000). In brief, 100µl of each extract solution (2.5mg/ml) was mixed with 1ml of Folin reagent (Thermo-fisher, India). After standing for 3 minutes, 0.8 ml of 1M Sodium Carbonate was mixed and shaken. The mixture was allowed to stand for 1 hour and the absorbance was measured at 765

nm. The calibration curve was prepared using Gallic acid as the standard of concentrations 25 mg/l, 50 mg/l, 75 mg/l, 100 mg/l, 125 mg/l, 150 mg/l, 175 mg/l, 200 mg/l, 225 mg/l and 250 mg/l. Total phenol values were expressed as mg GA equivalent per gram dry extract weight.

Determination of total flavonoid content

Aluminum chloride colorimetric method as described by Gao (2000) was used for flavonoids determination with some modification. In brief 0.25ml of each extract solution (10mg/ml) was mixed with 1.4 ml of distilled water. 0.75 ml of methanol was added then 0.005 ml of 1M Potassium Acetate was added. Then after 5 minutes, 0.005 ml of 10% Aluminum Chloride was added .The mixture was shaken and the absorbance was measured at 415 nm using UV spectrophotometer. The calibration curve was prepared using quercetin as the standard of concentration 10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l, 50 mg/l, 60 mg/l, 70 mg/l, 80 mg/l, 90 mg/l and 100 mg/l. Total flavonoid values were expressed as mg quercetin equivalent per gram dry extract weight.

Determination of antioxidant activity using 2, 2- diphenyl-1-picrylhydrazyl free radical

Antioxidant activity of the cheese extracts was assayed using DPPH free radical (Kim et al., 2007) with modification. DPPH solution (0.2 mM) was prepared by dissolving 7.886 mg of DPPH in 100 mL methanol and Stirred overnight at 4°C. Thus prepared purple colored DPPH (Thermo-Fisher, India) free radical solution was stored at -20°C for further use. Different concentrations (100, 200, 300, 400, 500, 600, 700 and 800 µg/ml) of methanolic solutions of each extracts were prepared by the serial dilution of the stock solution (10 mg/ml) of the respective extract. To each 0.5 ml extract Solution, 0.5 ml of 0.2 mM DPPH solution was added. A control was prepared by mixing 0.5 ml methanol and 0.5ml 0.2 mM DPPH solution. These samples were shaken well and kept in dark for 30 minutes at room temperature. The absorbance was measured at 517 nm against the blank solution consisting MeOH. The radical scavenging activity was expressed as the radical scavenging percentage using the equation.

% Radical Scavenging Activity= [(Control Abs- Sample Abs)/ Control Abs] x100

IC50 value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the graph of radical scavenging activity against the concentration of extracts (Fig A).

Antibacterial activity of phytochemicals by agar well diffusion method

Sensitivity of bacterial strains to various phytochemical extracts of cheese and plants were measured in terms of Zone of inhibition using agar well diffusion assay as described by Shakouie et al, 2012. Bacterial strains used in this study such as *Escherichia coli* and *Staphylococcus aureus*. The bacterial cultures were grown in Muller Hinton Agar (HI media, India) and Muller Hilton Broth. The sterile plates containing Muller-Hilton agar medium were spread with fresh bacterial culture by using sterile cotton buds. Well (5 mm size) were made from agar plates by using sterile corkborer, the wells were loaded with 30 µl of phytochemical extracts of concentration (25, 50 and 100 mg/ml). The plates were incubated at 37°C for 24 hours. After, inhibition the plates were observed for the presence of clear inhibition zone around the well. The zone of inhibition were calculated by measuring the diameter of the inhibition zone around the well. Methanol and Ampicillin were used as controls.

4. Brine Shrimp Lethality Bioassay of Cheese Extract

Hatching the brine shrimp

Brine shrimp eggs (*Artemia salina*) were hatched in artificial salt water prepared from salt (40 g/1) and supplemented with 6 mg/1 dried yeast. The two unequal compartments plastic chamber with several holes on the divider was used for hatching. The eggs were sprinkled into the larger compartment which was darken, while the smaller compartment was illuminated. After 48 hours incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighted side whereas their shells were left in another side.

Bioassay

The procedure for BSLT was modified from the assay described by Solis et al. (1993). Stock of extract of concentration 2.5 mg/mL was made and serial dilution was carried upto 10^{-1} and 10^{-2}. 100µl of respectively each of stock and the serially diluted test extract were placed in the wells of 96-well microplates. 100µl of suspension of nauplii containing 10-15 organisms was added to each well so that the final concentration was 1000 µg/ml, 100 µg/ml and 10 µg/ml. Potassium dichromate was used as positive control and salt water with shrimp only was taken as negative control. The plates were covered and incubated at room temperature (25-29°C) for 24 hours. Plates were then examined under the binocular stereomicroscope and the numbers of dead (non-motile) nauplii in each well were counted. 100 µl of methanol were then added to each well to

immobilize the nauplii and after 15 minutes the total numbers of brine shrimp in each well were counted. The percentage mortality (% M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts. The lethal concentrations of cheese extract resulting in 50% mortality of the brine shrimp (LC50) from the 24 h counts was calculated from the graph of % mortality against the log of concentration of extracts (Fig B).

Statistical analysis

All experiments were carried out in triplicates. One way and two way ANOVA was carried out for data analysis at 0.05 level of significance using Microsoft Office Excel 2010.

5. Results and Discussion

The yield of extract in methanol ranged 6.67-7.77 % for cheese considering 20% moisture content (Table A).The *nak* cheese extract were yellowish compared to extract of cow cheese. Yellow color of cheese extract corresponds to concentration of beta-carotene dissolved in fat, which is associated with exposure to herbage and silage of pasture land as well as temperature dependent denaturation (Martin et al., 2005). The significant yellow coloration of *nak* cheese extract indicates the long-term exposure of *nak* to green vegetation of higher altitudes on average than cow. Cows are mostly fed with grain based diet for high milk production and are given certain variety of green fodder. Cows spend most of their time tied up in the farms. Whereas *nak* forage freely in the open pastures of higher altitudes and encounter different types of green grass and plants; most of them with high medicinal importance. The results are similar to the findings of Coppa, Verdier-Metz I and Ferlay who have suggested the presence beta- carotene of 100 % grass-fed whole milk to be 4 times higher than the level in milk from conventionally fed cows.

Table A: Extract yield (%) of cheese sample in methanol.

Sample	Weight of sample taken(g)	Yield (%)
Nak cheese (2400 m)	35	6.67 ± 0.1^{d}
Nak cheese (2900 m)	35	7.13 ± 0.2^{c}
Nak cheese (2600 m)	35	7.77 ± 0.1^{a}
Cow cheese (1900 m)	35	7.62 ± 0.2^{b}

Each Value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$).

Table B: Antibacterial activity of cheese extract against *E.coli* and *S. aureus*.

Extract Concentration(100mg/ml)	Test organisms	
	E. coli	*S. aureus*
Nak cheese (2400 m)	-	-
Nak cheese (2900 m)	-	-
Nak cheese (2600 m)	-	-
Cow cheese (1900 m)	-	-

Table C: Total phenol content (TPC), Total flavonoid content (TFC), antioxidant activity (IC_{50}) and toxicity (LC_{50}) of cheeses extract

Sample	TPC (mg GA/g)	TFC (mg QE/g)	IC50 (mg/L)	LC50 (µg/mL)
Nak cheese (2900 m)	10.9± 0.7[d]	7.70±0.5[d]	371.64±3.3[d]	698.95±4.3[a]
Nak cheese (2600 m)	9.79±0.8[e]	5.45[e]±0.6[e]	394.12±3.1[c]	411.18±6.1[b]
Nak cheese (2400 m)	6.06± 0.6[f]	4.12±0.4[f]	518.30±2.7[b]	350.19±4.6[d]
Cow cheese (1900 m)	2.75± 0.4[g]	1.80±0.3[g]	626.24±4.2[a]	376.84±5.7[c]

Each value is the average of three values ± standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$)

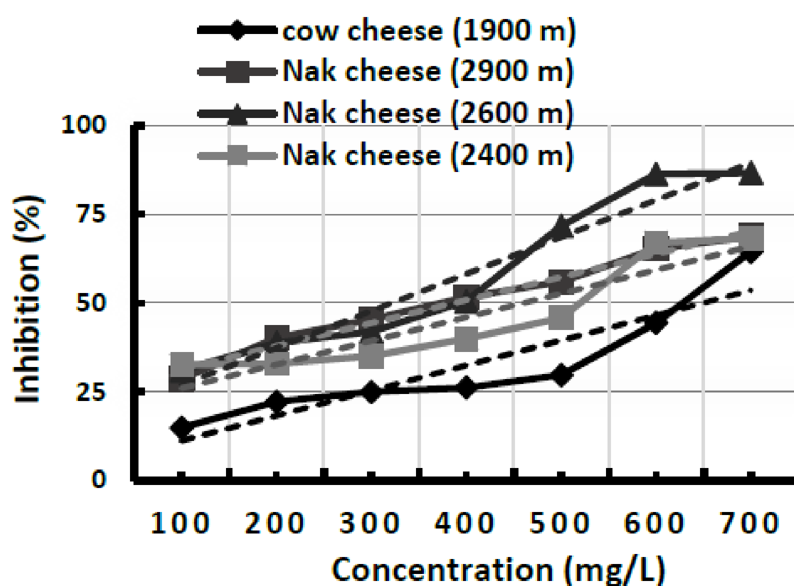

Figure A: DPPH radical scavenging activity of cheese extract at wavelength of 517 nm.

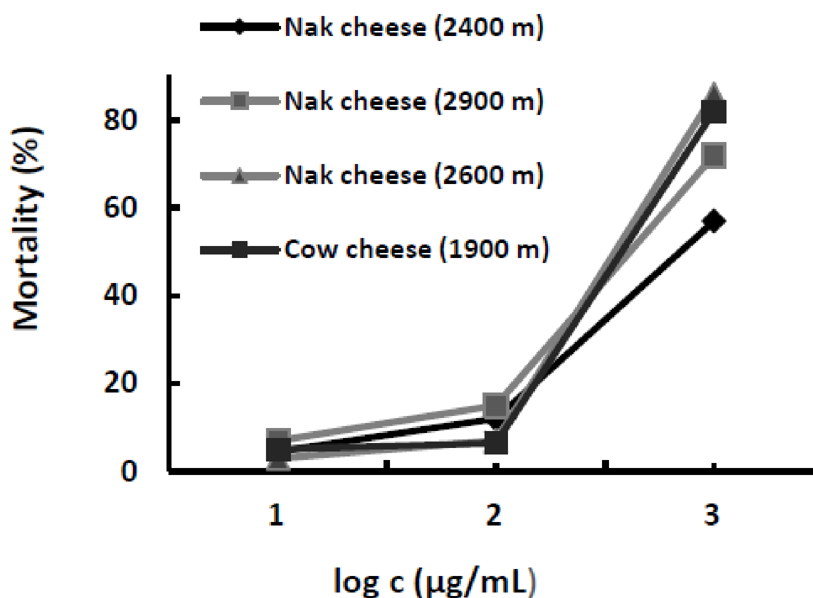

Figure B: Brine shrimp lethality bioassay of cow and *nak* cheeses extracts at 24 h

Phenol and flavonoid can influence milk and cheese taste and can also affect their antioxidant activity. Thus, it was reasonable to determine their total amount in the plants and cheeses extract. The presence of phenolic compounds in the milk and later in the cheese might be a result of their transfer from plant to milk (Hilario et al., 2010). Comparatively, *nak* cheese had higher content of phenol and flavonoid than cow cheese (Table C), which may be due the difference in their feeding habit, domestication and duration of exposure to green vegetation. The results are also supported by previous study of Hilario et al, 2010, that evaluated the presence of bioactive polyphenol compounds in milk of goats grazing on shrubby rangeland vegetation and full-indoor confinement. In the study, he showed that TPC was affected by animal feeding, and its value was higher in the milk of grazing goat than in the milk of indoor goat. In recent years. Several studies have shown that some plant compounds (e.g. vitamins, volatile organic compounds) are directly transferred from the grazed herbage to the milk (Fedele et al., 2000: Pizzoferrato et al., 2000). Connel and Fox, 2001 reported that the majority of phenolic compounds found in cow milk are derived from the feed and when the cows are fed large quantities of particular crops. However, the phenols and flavonoids detected in the cheeses might also be produced by secondary microflora of cheese, which may consists of fungus and molds besides LAB. Certain class of flavonoids are produced by fungus as secondary metabolites. Similarly fungi have genes encoding the production of volatile phenols (Chatonnet et al, 1995).

The antioxidant activities of the prepared cheese extracts were investigated by DPPH scavenging assay as *2, 2-Diphenyl-2-picrylhydrazyl Hydrate (DPPH) Radical Scavenging Assay* (Table C) has been widely

used for the antioxidant activity evaluation of food and biological samples. The ability of these extracts to scavenge DPPH suggest that they might have antioxidants that react with free radicals to convert them to more stable products and terminate radical chain reaction. The increased antioxidant activity corresponding to increased phenol content indicates that phenol may also contribute to antioxidant activity of cheese along with antioxidant peptides in cheese present through raw milk or produced during ripening process by the microflora (McIntosh et al., 1995). Researcher Djeridane et al., 2006; Katsube et al., 2004 have shown positive correlation between phenol content and antioxidant activity. While others (Czapecka et al., 2005) show poor linear correlation or report total antioxidant activity and phenolic content with no comment. With no clear understanding of flavonoids as antioxidant or the mediators of signaling pathway (Williams et al., 2004), it seems irrelevant to associate flavonoid content of cheese extract to their antioxidant activities. These assay indicate antioxidant activity of *nak* cheese possibly due to the presence of phytochemicals or antioxidant proteins of the cheese and those produced by secondary microflora of cheese might be helpful in preventing the progress of various oxidative stresses leading to symptoms such as headache, nausea, anorexia, fatigue and lassitude. Regular consumption of *nak* cheese may also help to reduce the risk of oxidant-induced carcinogenesis. *Nak* milk and cheese has become an important ingredient of diet for the people residing in higher altitudes to cope with oxidative stress brought about by direct UV exposure and rarefaction of the atmosphere and has helped them to stay healthy and live longer (Askew, 2002). The presence of antioxidants in higher amount in *nak* cheese might also be a possible explanation for its longer shelf-life of over 4 month compared to cow cheese only ripened for a month, whereby higher concentration of antioxidants in nak cheese prevents considerable oxidative deterioration of the cheese.

The brine shrimp lethality assay has been used routinely in the crude extracts of plant and food samples to assess the toxicity towards brine shrimp, which provides an indication of possible cytotoxic and anti-tumor properties of the test materials (Peteros and Yu, 2010). The variation in BSLA (Table C) results may be due to the difference in the amount and kind of cytotoxic substances (e.g. flavonoids or phenols), the dietary proteins such as casein and whey and secondary microbial metabolites in the crude extracts of the cheese. As mentioned by Meyer and others, LC50 of less than 1000 µg/mL is toxic while LC50 of greater than 1000 µg/mL is non-toxic. According to the toxicity scale of Hodge and Sterner, LC50 value 0f 100-1000 is considered moderately toxic**.** Therefore considering scale given by both the researchers, the cheese studied are moderately cytotoxic**.** The moderate lethality of several cheese extracts to brine shrimp is an indicative of the presence of potent cytotoxic components and probably insecticidal compounds (Riser and Cortes, 1996) which may have effects on both normal and cancerous cells. Yasuda et al, 2013 reported lower cytotoxic effect of Pouligny Saint-Pierre cheese in differentiated HL-60 cells than undifferentiated HL-60 cells at the varying

concentrations tested. cheese demonstrated higher cytotoxicity through anti-proliferative activity.Based on the results of such experiments and the results of this study; consuming *nak* cheese regularly in low dose might not have serious health implications but may exhibit anti-cancerous effects.

In the present study, antibacterial activity cheese extracts were investigated against two bacterial species (*Staphylococcus aureus* and *Escherichia coli*) at concentration of 25, 50 and 100 mg/mL (Table B). Cheeses extract showed no conspicuous zone of inhibition at all three concentration in both the pathogens. The result indicates that cheese extract even if they have phytochemicals and antimicrobial proteins, their presence in low concentration and the bacteria and pH-dependent transformation of antioxidants antimicrobial activity or denaturation of phytochemicals or antimicrobial proteins during pasteurization may be the probable cause of failure to show antimicrobial activity. According to Bingham 2006, Phytochemicals can be transformed by cheese and intestinal micro flora can or metabolize some polyphenols compounds to catechol and other simple phenols; and the derivatives formed in these reactions are characterized by different antimicrobial activities comparing to the precursors. The result suggest that cheese are prone to spoilage pathogens, so there is a need of controlling contamination during cheese production and ripening stages.

5. Conclusion

Nak cheese can be considered a cheese with good source of antioxidants. The cheese extracts showed moderate cytotoxicity towards the brine shrimp used in bioassay, probably due to the presence of bioactive molecules such as phytochemicals or dietary proteins in considerable amount, therefore necessitates assay of possible health effects of consuming nak cheese. The results of this study supports the hypothesis that pharmaceutically important phytochemicals may be present in *nak* cheese with the feeding of medicinal plants of higher altitudes by the *nak*. This study also shows that content of bioactive components is affected by the animals feeding system. Longer extent of grazing provided an increase of total polyphenol, and flavonoid concentrations in nak cheese compared to cows. More research is needed to elucidate the potential of nak cheese as a functional food.

References

[1]. Alberto M.R., Farias M.E., Manca De Nadra M.C., (2001). Effect of Gallic acid and catechin on Lactobacillus hilgardii 5w growth and metabolism of organic compounds. J. Agric. Food Chem. 49 (9), 4359-4363.

[2]. Bingham M., (2006). The metabolism of polyphenols by the human gut microbiota. In: Gastrointestinal microbiology. Eds A.C. Ouwehand, E.E Vaughan. Taylor & Francis, New York, 155-168.

[3]. Bovolenta S, Corazzin M, Sacca E, et al (2009). Performance and cheese quality of Brown cows grazing on mountain pasture fed two different levels of supplementation. Livestock Science, Volume 124, Issues 1-3, Pages 58-65.

[4]. Coppa M, Verdier-Metz I, Ferlay A, et al (2011).

[5]. Effect of different grazing systems on upland pastures compared with hay diet on cheese sensory properties evaluated at different ripening times. International Dairy Journal, Volume 21, Issue 10, Pages 815-822.

[6]. De Feo V., Quaranta E., Fedele V., Claps S., Rubino R. and Pizza C (2006), Flavonoids and terpenoids in goat milk in relation to forage intake, Italian Journal of Food Science., 1, 85-92.

[7]. E. W. Askew, (2002). Work at high altitude and oxidative stress: antioxidant nutrients. Journal of Toxicology, vol. 180, no. 2, pp. 107-119.

[8]. G.Mohan, S.P.Anand, A.Doss, et al (2011). Efficacy of Aqueous and Methanol extracts of Caesalpinia Sappan L. and Mimosa pudica L. for their potential Antimicrobial activity. South Asian Journal of Biological Science. 1(2): 48-57, Research Article (ISSN 2249 – 6599).

[9]. Hilario M.C., Puga C.D. Ocana A.N. and Romo F.P.G., Antioxidant activity, bioactive polyphenols in Mexican goats' milk cheeses on summer grazing, Journal of Dairy Research., 2010, 77, 20-26.

[10]. Hitti, Miranda (2008). Cheese From Grass-Fed Yaks May Have Better Fatty Acid Profile Than Cheese From Grain-Fed Cows, Study Shows. WebMD Health News.

[11]. Miller, D.J. (1987). Yaks and grasses: pasturalism in the Himalayan countries of Nepal and Bhutan and Strategies for sustained development. UNDP.

[12]. Pande, R.S. (1996). Livestock development: In search of greener pastures. The Rising Nepal. October 4, 1996.

[13]. Pande, R.S. (2004). Chauri Production systems in Upper Slopes Areas, Sindhupalchok, Nepal. Fourth International Congress on Yak, September 20-26, Chngdu, China.

[14]. Saif Alyaqoubi, Aminah Abdullah , Muhamad Samudi, Norrakiah Abdullah , Zuhair Radhi1Addai, Maryam Al-ghazali (2015). Physicochemical Properties and Antioxidant Activity of Milk Samples Collected from Five Goat Breeds in Malaysia, Advance Journal of Food Science and Technology 7(4): 235-241, ISSN: 2042-4868; e-ISSN: 2042-4876.

[15]. S. Bovolenta, A. Romanzin, M. Corazzin, M. Spanghero, E. Aprea, F. Gasperi, E. Piasentier (2014). "Volatile compounds and sensory properties of Montasio cheese made from the milk of Simmental cows grazing on alpine pastures. Journal of dairy science, Volume 97, Issue 12, Pages 7373-7385.

[16]. Shah NP, Lankaputhra WEV, Britz ML, Kyle WSA (1995) Survival of Lactobacillus acidophilus and Bifidobacterium bifidum in commercial yoghurt during refrigerated storage. International Dairy Journal 5: 515-521.

[17]. Solis, P. N., Wright, C. W., Anderson, M. M., Gupta, M. P., & Phillipson, J. D. (1993). A microwell cytotoxicity assay using Artemia salina (brine shrimp). Planta Medica, 59, 250-252.

[18]. S. Yasuda, N. Ohkura, K. Suzuki, M. Yamasaki, K. Nishiyama, H. Kobayashi, Y. Hoshi, Y. Kadooka and K. Igoshi (2010), “Effects of Highly Ripened Cheeses on HL-60 Human Leukemia Cells: Antiproliferative Activity and Induction of Apoptotic DNA Damage,” Journal of Dairy Science, Vol. 93, No. 4, pp. 1393- 1400.

[19]. Williams RJ, Spencer JP, Rice-Evans C (2004). Flavonoids: antioxidants or signaling molecules?, Free Radical Biological Medicine; 36(7):838-49.

[20]. Winter J., Moore L.H., Dowell V.R., Bokkenheuser V.D. (1989). C-Ring cleavage of flavonoids by human intestinal bacteria. Applied and Environmental Microbiology. 55 (5), 1203.